AD_____

Award Number: DAMD17-01-1-0346

TITLE: Antiangiogenic Action of Chemically Modified Tetracyclines in Breast Cancer

PRINCIPAL INVESTIGATOR: Sanford R. Simon, Ph.D.

CONTRACTING ORGANIZATION: State University of New York
Research Foundation
Stony Brook, New York 11794-3362

REPORT DATE: June 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual (15 May 03 - 14 May 04)

4. TITLE AND SUBTITLE
Antiangiogenic Action of Chemically Modified Tetracyclines in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0346

6. AUTHOR(S)
Sanford R. Simon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
State University of New York Research Foundation
Stony Brook, New York 11794-3362

E-Mail: ssimon@notes.cc.sunysb.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The long term objective of this project remains to evaluate nonantimicrobial chemically modified tetracyclines (CMTs) as inhibitors of the angiogenic response which maintains breast tumor growth and proliferation. In this third year, we expanded our studies on release of VEGF from breast tumor cell lines to include a fourth cell line, MDA-MB-435s, which is more invasive than MCF-7: CMT-300 stimulated release of VEGF from both cell lines except at the high dose of 50 uM, but CMT-308 inhibited release from both cell lines in a dose dependent fashion. TGF-beta augmented VEGF release by both cell lines, but this enhanced release was also inhibited by CMT-308 in a dose-dependent fashion. Neither CMT triggered cytotoxicity in either line at doses up to 50 uM. All CMT-induced diminution of released VEGF levels could be ascribed to inhibition of synthesis and/or secretion, rather than degradation of pre-existing growth factor. The steady-state levels of intracellular VEGF species (via Western blot) and mRNAs for these species (via PCR) were unaffected by TGF-beta or CMTs, suggesting primary effects on the secretory pathway. In contrast, intracellular pools of VEGF in Mono Mac 6 cells were diminished by CMT-308, although by far less than the inhibition of secretion; there was no significant effect of CMTs on the multiple VEGF mRNA species. Both CMT-300 and CMT-308 visibly reduced tube formation by human microvascular endothelial cells on collagen, although by less than when cells were plated on Matrigel. These results offer further support for our argument that CMTs may be of use as antiangiogenic agents in management of breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Angiogenesis, tetracyclines, macrophages, breast tumor cell lines, endothelial cells, vascular endothelial growth factor	37
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

A. Introduction

We have maintained our focus on the long term objective of this project: to evaluate the capacity of a group of nonantimicrobial chemically modified tetracyclines (CMTs) to downregulate the angiogenic response which maintains breast tumor growth and proliferation. Our strategy is based on two potential modes of action of these agents. Breast tumor angiogenesis *in vivo* is correlated with the capacity of the tumor cells as well as infiltrating macrophages to release soluble pro-angiogenic factors, including Vascular Endothelial Growth Factor (VEGF), which induce endothelial cell proliferation and differentiation into structures which assume the characteristics of neovessels. Based on observations of *in vivo* antiangiogenic activity of 6-deoxy-6-demethyl-4-de(dimethylamino)tetracycline (CMT-300) in patients with Kaposi's sarcoma, we have hypothesized that the CMTs could downregulate VEGF production, could inhibit endothelial cell neovessel formation, or could act by a combination of these two modes of action. Clinical trials of CMT-300 in management of Kaposi's sarcoma have progressed to Phase II studies, and continue to offer promising results [1]. Reports on antivasculogenic and antiangiogenic effects of the CMTs *in vitro* offer further support for the potential application of these agents as inhibitors of breast tumor angiogenesis [2,3]. While CMT-300 continues to show a high level of safety, not only in NCI-sponsored clinical trials on refractory cancer patients but also in Phase I trials in normal human volunteers and in patients with rosacea, we recognize that this agent resembles tetracycline antibiotics in its capacity to produce phototoxicity in some patients who are exposed to sunlight without sunscreen protection [4]. In contrast, the 9-amino derivative of CMT-300 (CMT-308) shows minimal phototoxicity *in vitro* and in animal models, and has been evaluated in this project in parallel with its parent compound for its capacity to inhibit release of VEGF and to block neovessel formation by endothelial cells. In this annual report we summarize our further progress in characterizing the effects of these two CMTs on release of pro-angiogenic factors by a current total of four human breast tumor cell lines and a monocytoid line, and on the capacity of human endothelial cells to undergo a shape change analogous to the formation of so-called "tubes" when plated on type I collagen or when attempting to migrate towards VEGF as a chemoattractant.

B. Body

This project remains focused on six Tasks which we established for the original three year and now four year duration of support: to evaluate CMTs for effects on angiogenic factors released by breast tumor cell lines maintained under "basal" growth conditions (Task 1); to evaluate modulation of angiogenic factor release from the tumor cell lines by hormones, cytokines, and growth factors and to determine effects of the CMTs on these modulated levels of angiogenic factor release (Task 2); to evaluate effects of the CMTs on release of angiogenic factors by macrophages and a human macrophage cell line, Mono Mac 6 (Task 3); to evaluate capacity of the CMTs to inhibit tube formation and invasiveness of endothelial cells in response to pro-angiogenic factors (Task 4); to evaluate capacity of the CMTs to inhibit endothelial cell tube formation and invasiveness in response to breast tumor cell lines in a co-culture system (Task 5); and to evaluate capacity of the CMTs to inhibit endothelial cell tube formation and invasiveness in response to monocytoid cells in a co-culture system (Task 6). In the third year of the project we have made additional significant progress primarily on the first four of these Tasks and have refined assays to address the last two Tasks.

1. Task 1 - Effects of CMTs on VEGF Release by Breast Tumor Lines in the Absence of Growth Factors

In the first two years we demonstrated that CMT-308 was more effective than CMT-300 in diminishing the levels of VEGF released by three breast tumor cell lines, MCF-7, MDA-MB-231, and MDA-MB-453. MCF-7 is estrogen unresponsive, poorly invasive *in vitro*, and minimally metastatic in animal models, whereas MDA-MB-231 is estrogen unresponsive, highly invasive and highly metastatic. MCF-7 is thus often regarded as a model of early breast cancer whereas MDA-MB-231 is regarded as a model of highly aggressive or advanced metastatic disease. The third cell line we examined, MDA-MB-453, is estrogen unresponsive and expresses the HER-2/*neu* oncogenic receptor, like MDA-MB-231, but has very low tumorigenicity in animal models; it is significantly less invasive than MDA-MB-231, but more invasive than the estrogen-responsive, HER-2/*neu*-negative, MCF-7 line *in vitro* [5]. This year, we added the MDA-MB-435S cell line, which is closer to MDA-MB-231 in its invasiveness and tumorigenicity to our panel. MDA-MB-435S cells are also estrogen receptor negative and, like MDA-MB-231 cells, highly invasive and metastatic in nude mouse models [5,6]. Using the same culture protocol as we had established for the previous three cell lines, we found that MDA-MB-435S cells released about the same levels of VEGF as MCF-7 cells in the absence of inhibitors or growth factors (~300 pg/ml per 10^5 cells over ~16 hours for MCF-7 vs. ~270 pg/ml per 10^5 cells over 16 hours for MDA-MB-435S) [**Figures 1** and **2**]. As we previously reported, at concentrations of CMT-300 $\geq$5 μM there was evidence of progressive cytotoxicity, but no comparable loss of viability was detected with the breast tumor cell lines at even higher levels. However, doses of CMT-300 as low as 5 μM triggered an increase in mitochondrial dehydrogenase activity in all the breast tumor cell lines tested, as detected by reduction of the tetrazolium salt MTS to its formazan. This increase in mitochondrial dehydrogenase activity suggests that CMT-300 may induce some nonspecific "activation" of the cells [**Figures 1** and **2**]. We previously showed that CMT-300 had a modest stimulatory effect on "basal" VEGF levels released by MCF-7 cells in the absence of exogenous growth factors. A clear dose-dependent stimulatory effect of CMT-300 was also detected when MDA-MB-435S cells were incubated with the tetracycline derivative [**Figure 1**]. In contrast, CMT-308 diminished basal VEGF release from MDA-MB-435S as well as MCF-7 cells in a dose-dependent fashion. At the lowest dose of CMT-308 tested, 1 μM, there was a reproducible but small stimulation of VEGF release, but at 5 μM and higher doses of this tetracycline derivative, VEGF levels declined until released VEGF levels were diminished by greater than 50% in MDA-MB-435S cells and by greater than 80% in MCF-7 cells in the presence of 50 μM CMT-308, with no evidence of cytotoxicity or mitochondrial dehydrogenase stimulation in either cell line [**Figures 1** and **2**]. These results are qualitatively consistent with our previously observed studies on MDA-MB-231 and MDA-MB-453 cells. We have confirmed the time course of VEGF release from MCF-7 cells in the absence and presence of CMT-308 over the first 16 hours after replacement with serum-free medium [**Figure 3**]. In our earlier results reported last year, we found that during this time in the absence of CMT-308, VEGF levels became detectable between 2 and 4 hours after replacement of the medium and rose progressively over the next 20 hours. In the presence of 20 μM CMT-308, VEGF levels remained below detectable levels for the first 8 hours, and only began to rise sometime over the next 8 hours. Moreover, if the CMT was introduced at 8 hours, further

release of VEGF was suppressed for an additional 8 hours. The apparent potency of CMT-308 as an inhibitor of VEGF release is therefore a function not only of inhibitor dose but also of time in the presence of the agent.

2. Task 2 - Effects of Growth Factors on VEGF Release by Breast Tumor Cell Lines in the Absence and Presence of CMTs

We reported in the first and second annual progress reports that TGF-β, but not IGF-1, appeared to enhance VEGF release by the three breast tumor cell lines we had studied so far. This observation was extended to MDA-MB-435S cells as well. The enhancement of VEGF release from MCF-7 cells by TGF-β was monotonically dose-dependent, ranging from ~70 pg/ml per 10^5 cells in the absence of the growth factor to ~110 pg/ml per 10^5 cells in the presence of 10 ng/ml TGF-β, the highest dose tested [**Figure 4**]. At doses of TGF-β between 0.1 ng/ml and 10 ng/ml, VEGF levels released by MDA-MB-435S cells were also increased much more dramatically in a dose-dependent fashion, with four- to five-fold increases measured at 1 ng/ml and 10 ng/ml [**Figure 5**].

CMT-300 consistently produced a significant dose-dependent stimulation of VEGF release from MCF-7 cells in the presence of all doses of TGF-β tested, while it produced a more modest dose-dependent stimulation of VEGF release from MDA-MB-435S cells at the lowest dose of TGF-β tested, 0.1 ng/ml, and variable diminution in released VEGF levels at higher doses of TGF-β [**Figures 4** and **5**]. In contrast to CMT-300, 20 μM CMT-308 inhibited VEGF release from MCF-7 cells by ≥50% in the presence of all doses of TGF-β tested [**Figure 4**]. In the presence of 0.1 ng/ml or 1.0 ng/ml TGF-β, release of VEGF from MDA-MB-435S cells was also inhibited by significantly greater than 50% when cells were maintained in 20 μM CMT-308, although the efficacy of this agent appeared to be somewhat reduced at the highest dose of the growth factor, 10 ng/ml [**Figure 5**]. This pattern of responsiveness is similar to that which we had previously observed in MDA-MB-453 cells.

In this third year of the project, we have investigated further whether the effects of CMTs and TGF-β on release of VEGF by breast cancer cell lines can be traced to altered levels of mRNA species for the angiogenic factor, and we have also considered whether alterations in the size of the intracellular pools of the intracellular species of VEGF might be produced by these tetracycline derivatives. Intracellular VEGF species were visualized by Western blots, using intracellular actin levels to normalize the concentrations of intracellular protein from the different cell lysates, while PCR was used to detect species of VEGF mRNA, using levels of the mRNA for glyceraldehyde-3-phosphate dehydrogenase to normalize the samples from the different cell lines. Incubation with either 5 μM CMT-300 or 20 μM CMT-308 had no effect on the levels of immunoreactive intracellular VEGF species detected in lysates of MCF-7 cells [**Figure 6**] or MDA-MB-435S cells [**Figure 7**], either in the absence or presence of 1 ng/ml TGF-β. This observation contrasts with the significant augmentation by CMT-300 and diminution by CMT-308 of VEGF levels released into the surrounding medium by the two cell lines. Multiple isoforms of intracellular VEGF protein could be visualized in lysates of MCF-7 cells, whereas a single 46 kDa isoform could be detected in MDA-MB-435S lysates. These lysates were carefully prepared by multiple freeze-thaw cycles in the presence of a protease inhibitor cocktail to ensure that no artifactual proteolytic modifications might occur during lysis.

To detect VEGF mRNA species, we employed primer pairs which can be used to visualize three different VEGF transcripts, coding for a 189 amino acid species (479 base pairs),

a 165 amino acid species (407 base pairs), a 154 amino acid species (347 base pairs), and a 121 amino acid species (275 base pairs) [7]. Neither TGF-β nor a range of doses of CMT-308 had any effect on levels of the multiple VEGF mRNA species which could be detected in MCF-7 cells (predominantly the species coding for the 121 amino acid and 165 amino acid isoforms) [**Figure 8**]. Similarly, CMT-300 did not affect VEGF mRNA levels in MCF-7 cells, in the presence or absence of TGF-β [**Figure 9**]. This insensitivity of the levels of the dominant 407 bp and 275 bp species of VEGF mRNA to CMTs or TGF-β was also observed in MDA-MB-435S cells [**Figure 10**]. Based on these observations, we hypothesize that the most likely effects of CMTs and TGF-β on VEGF production by breast cancer cell lines are post-transcriptional and post-translational, perhaps at the level of secretion.

3. Task 3 - Effects of CMTs on VEGF Release by Mono Mac 6 Cells

We have continued to use the human monocytoid cell line Mono Mac 6 as a model for macrophages which infiltrate tumors *in vivo*. Using a protocol similar to that employed with the tumor cell lines, in which the cells are transferred to serum-free medium in the presence or absence of inhibitors and/or growth factors and supernatant medium is collected after 24 hours for VEGF quantitation by ELISA, we have confirmed that CMT-308 is a potent inhibitor of VEGF release from Mono Mac 6 cells, whereas CMT-300 appears to have a significant stimulatory effect on VEGF release at those low doses (≤5 μM) which cause less than 50% cytotoxicity. We have already presented results showing that CMT-300 appears to stimulate mitochondrial dehydrogenase activity of breast tumor cell lines at doses as low as 5 μM, as detected by MTS reduction to its formazan [*cf.* **Figures 1** and **2**]. At this low dose, significant cytotoxicity to Mono Mac 6 cells was already apparent (~40% loss of mitochondrial dehydrogenase activity). Higher doses of CMT-300 produced more cytotoxicity, which seemed to level off at ~50% [**Figure 11**]. We have speculated that the stimulation of VEGF release from Mono Mac 6 cells by 5 μM CMT-300 may be related to an "activation response" which may also occur in the breast tumor cell lines. We have further proposed that the apparent diminution of VEGF release from Mono Mac 6 cells at higher CMT-300 doses may be related to cytotoxicity, but may also reflect additional effects of the CMT on secretion. In contrast to CMT-300, CMT-308 caused less than 20% cytotoxicity to Mono Mac 6 cells at the highest dose tested (20 μM), but at 5 μM, CMT-308 had already suppressed VEGF release from Mono Mac 6 cells by ~50%, and at 20 μM CMT-308, VEGF levels in the extracellular medium had fallen to the lower limit of detection [**Figure 11**]. When we followed the time course of VEGF release from Mono Mac 6 cells after transfer to fresh serum-free medium, we could detect measurable levels of VEGF after ~4 hours, which continued to rise over the next 20 hours [**Figure 12**]. In the presence of 20 μM CMT-308, however, VEGF levels in the supernatant medium of Mono Mac 6 cells remained below the limit of detection over the entire 24 hour study period. In results not shown here, we have observed continued suppression of VEGF release from Mono Mac 6 cells by CMT-308 for as long as 48 hours after introduction of the tetracycline derivative. This result contrasts with the apparent capacity of the breast tumor cell lines to "escape" from the suppressive effects of CMT-308 after 8 hours of exposure. In the first year of this project, we established that VEGF release, and the effects of the CMTs on levels of released VEGF, are completely insensitive to TGF-β and IGF-1. We have since confirmed that neither the augmentation of VEGF release in the presence of 5 μM CMT-300 nor the virtually total inhibition of VEGF release by 20 μM CMT-308 are affected by doses of TGF-β up to 10 μM [**Figure 13**].

As described above for our studies on breast tumor cell lines, we have employed Western blotting of Mono Mac 6 cell lysates (again using actin levels to normalize the intracellular protein concentrations in samples from cells cultured under different conditions) and PCR of total mRNA preparations (normalized to the levels of the mRNA for glyceraldehyde-3-dehydrogenase) to determine the effects of the CMTs on the levels of intracellular VEGF species and the mRNAs for those species. Like MCF-7 cells, but unlike MDA-MB-435S cells, Mono Mac 6 cells appeared to produce intracellular VEGF species of higher molecular weights than the 46 kDa form, which was also detected in the lysates [**Figure 14**]. Neither 5 μM CMT-300 nor 20 μM CMT-308 had any dramatic effect on the intracellular levels of the higher molecular weight isoforms in lysates of Mono Mac 6 cells, although there appeared to be significant diminution in the levels of the intracellular 46 kDa species in lysates of CMT-300-treated cells. In contrast to the profile of intracellular species, the levels of the secreted higher molecular weight isoforms (the only species which could be visualized in the extracellular medium) were virtually undetectable in the medium from Mono Mac 6 cells which had been cultured in the presence of CMT-308, consistent with the ELISA measurements of secreted VEGF described above. These higher molecular weight isoforms were, however, detected by Western blotting in the extracellular medium of cells maintained in the presence of CMT-300, again consistent with the results of the ELISA measurements [**Figure 14**].

Using the primers we had employed for visualization of VEGF mRNA species in breast tumor cell lines, we were able to detect the same species coding for a 165 amino acid isoform and a 121 amino acid isoform that we had seen in MCF-7 and MDA-MB-435S cells. Even after normalization to levels of the mRNA for glyceraldehyde-3-phosphate dehydrogenase, the levels of the two VEGF mRNA species from cells cultured in the presence of 5 μM CMT-300 appeared to be visibly diminished [**Figure 15**]. It should be noted that of all the cells studied so far in this project, only CMT-300-treated Mono Mac 6 cells experience significant diminution in the conversion of MTS to its formazan. In contrast, culture in the presence of 20 μM CMT-308 had no effect on the levels of the two VEGF mRNA species, consistent with the minimal effects of CMT-308 on intracellular VEGF protein isoforms [**Figure 15**]. We conclude that CMT-308 is an inhibitor of VEGF release, not only in breast tumor cells lines, but also in a monocytoid cell line. This effect also appears to be at a post-transcriptional and post-translational level in Mono Mac 6 cells, and may involve some step in the secretory mechanism.

4. Task 4 - Effects of CMTs on Human Endothelial Cells

In this third year of the project, we have made further progress in providing the experimental support for a key element of our initial hypothesis: that the antiangiogenic activity of CMTs may arise from two mechanisms - inhibition of pro-angiogenic factor release from tumor cells and infiltrating macrophages and prevention of neovessel formation by endothelial cells. Last year we described results with human microvascular endothelial cells plated onto 96-well microplates on which a thick layer of Matrigel™, a basement membrane-like extracellular matrix, had been applied. The growth factors which remain associated with Matrigel induce endothelial cells which are plated at modest densities to develop bridge-like extensions, or "tubes," from one small cell aggregate to another [8]. Both CMT-300 and CMT-308 were found to be potent inhibitors of tube formation from human dermal microvascular endothelial cells plated onto thick Matrigel layers. This inhibition was not associated with any visible evidence of cytotoxicity, as reflected in the intensity of fluorescence from calcein, which is generated from

its nonfluorescent precursor only in viable cells. In the absence of CMTs, the conditions we employed resulted in tubes which appear to be multicell structures bridging separated islands or clumps of cells. These tubes were significantly reduced in thickness by doses of CMTs as low as 1 μM, with a progressive reduction in the number of bridges which continued as somewhat higher doses of CMTs were employed. This loss of tube structures appeared to have reached at least 50% at doses of the CMTs around 10 μM and appeared to be complete at the highest doses of CMT-300 tested (30-50 μM). The diminution of tube structures in the presence of CMT-308 also appeared to plateau at doses around 30-50 μM, although a scant number of tubes appeared to persist at these high doses. In separate experiments with the tetrazolium salt MTS, we confirmed that the CMT-induced diminution in tube formation was not associated with cytotoxicity. We concluded that CMT-300 and CMT-308 effectively block tube formation from human endothelial cells on Matrigel with an approximate IC_{50} of 15 μM.

This year we employed a somewhat simpler assay described by Whelan and Senger [9] in which type I collagen is applied to cultures of endothelial cells grown on multiwell plates. As the collagen polymerizes at 37°C, it induces the cells to undergo a shape change, or morphogenesis, to form tube- or cord-like bridging structures, apparently triggered by selective interaction of integrins with the protein. We have continued to employ the combination of microvascular endothelial cells supplied by Cascade Biologics and endothelial growth medium supplied by Clonetics Corporation which have been recommended by B-D Biosciences for their tube-forming assays in Matrigel. Cells were maintained in 48 well plates for 24 hours in CMT-free medium or in medium containing 30 μM CMT-300 or CMT-308. The medium was then replaced with medium containing 500 μg/ml type I collagen purified from rat tail and the cells were cultured for an additional seven hours. At the end of the first hour, the collagen had polymerized, and tube formation ensued over the next six hours. As shown in **Figure 16**, islands of endothelial cells which had been cultured in the absence of CMTs were spanned by thick, multicellular bridges at the end of the culture period. In the presence of 30 μM CMT-300 or CMT-308, however, the bridges were reduced in number and appeared significantly thinner. This response to the two CMTs was qualitatively similar to, although not as pronounced as, the response of endothelial cells plated onto Matrigel we described last year. To see if the CMTs could reverse the formation of these cord-like bridges, the endothelial cells were cultured for seven hours with type I collagen in the absence of CMTs, and the medium was then replaced with fresh medium containing 30 μM CMT-300 or 30 μM CMT-308. As shown in **Figures 17** and **18**, the morphology of the cells which had been induced to undergo the morphogenic transition by collagen alone appeared to be unaffected by subsequent addition of CMT-300 and perhaps only slightly affected by CMT-308. We will be pursuing the nature of the effects of CMT-300 and CMT-308 on tube formation and collagen-triggered morphogenesis by endothelial cells further in the final year of this project.

In a further attempt to characterize the effects of CMT-300 and CMT-308 on endothelial cell function, we employed a migration assay from B-D Biosciences which we described last year as still being in developmental stages. This assay employs porous membrane-bottomed inserts for 24-well plates which have been fabricated with a fluorescence quenching dye within the membrane (Fluoro-Blok Inserts™) and which have been coated with the interstitial extracellular matrix protein fibronectin. The fibronectin coating does not completely occlude the pores of the inserts, so that cells applied to the top surface of the inserts reach the bottom surface by migration rather than invasion. Human umbilical vein endothelial cells were plated onto the

top surface of these inserts in medium alone or containing 10 μM CMT-300 or 20 μM CMT-308, while VEGF alone or with 10% fetal bovine serum was introduced into the surrounding medium below the inserts to serve as a chemoattractant. After 22 hours of culture, the inserts were transferred to 24-well plates containing calcein-AM and incubated for an additional 90 minutes. The fluorescence signal arising from endothelial cells which had migrated to the lower surface of the porous membranes was then quantitated with a Cytofluor 2300 microplate fluorimeter (fluorescence from cells remaining on the top surface of the inserts is blocked from the detector by the Fluoro-Blok membranes). VEGF alone stimulated migration of endothelial cells to the lower surface in a dose-dependent fashion, although not as effectively as in the presence of 10% fetal bovine serum. Migration of endothelial cells in response to VEGF alone was significantly inhibited by 10 μM CMT-300, but when VEGF + 10% serum was used as the chemoattractant, inhibition of migration by CMT-300 was more modest [**Figure 19**]. CMT-308 at a concentration of 20 μM was less effective than 10 μM CMT-300 in inhibiting migration of the endothelial cells in response to VEGF alone, and appeared to be ineffective at blocking migration in response to VEGF + serum [**Figure 20**]. These results are qualitatively consistent with the tube-forming assay results and suggest that the CMTs we have been studying may affect the capacity of endothelial cells to undergo the changes in shape that are essential for neovessel formation in addition to any other effects they may have on mechanisms of cell invasiveness.

5. Tasks 5 and 6 - Effects of Tumor Cell- and Macrophage-Derived Angiogenic Factors on Endothelial Cells and Inhibition of Endothelial Responses by CMTs

The studies we have proposed on induction of endothelial cell migration by co-culture with the MCF-7 and MDA-MB-435S breast tumor cell lines and Mono Mac 6 cells will be undertaken this coming year now that Fluoro-Blok inserts have become available from BD Biosciences. Similarly, we will evaluate the capacity of conditioned medium from 24 hour cultures of the tumor cell lines and Mono Mac 6 cells to enhance endothelial cell tube formation once BD Biosciences releases 96-well plates coated with Matrigel that has been sufficiently depleted of endogenous angiogenic factors to allow detection of enhanced tube formation by exogenously added pro-angiogenic factors.

C. Research Accomplishments - 5/15/03 - 5/15/04

- Characterized release of "baseline" levels of VEGF by a fourth human breast tumor cell line, MDA-MB-435S, previously described as a model of highly invasive breast cancer.
- Characterized dose-dependent enhancement of VEGF release from MDA-MB-435S cells by culture in the presence of TGF-β.
- Refined effects of CMT-300 and CMT-308 on release of VEGF from MCF-7 and MDA-MB-453 cells: CMT-300 at 5 μM stimulates VEGF release from MCF-7 cells and at doses up to 20 μM also stimulates VEGF release from MDA-MB-435S cells; in both cell lines this augmented release is accompanied by increased conversion of MTS to its formazan, suggesting an "activation response." CMT-308 inhibits release of VEGF from MCF-7 and MDA-MB-435S cells in a dose dependent fashion in the absence of an "activation response" or cytotoxicity. The inhibitory effect of CMT-308 appears to be somewhat blunted by very high TGF-β levels (10 ng/ml) in MDA-MB-435S cells, but in the presence of lower TGF-β levels, CMT-308 diminishes levels of secreted VEGF by

50% or more at doses which have been demonstrated to be achieved in the blood of human patients.

- Established time course of release of VEGF from MCF-7 cells and suppression of VEGF release by CMT-308: confirmed that newly released VEGF is first detected 2-4 hours after initiation of culture of MCF-7 cells in the presence or absence of TGF-β, and that 20 μM CMT-308 completely suppresses release of VEGF, also in the presence or absence of TGF-β, for up to 8 hours. After 8 hours of exposure to CMT-308, VEGF production is no longer suppressed. If the CMT-308 is introduced after 8 hours of culture in the absence of CMTs, no additional VEGF is produced over the subsequent 8 hours, but the pre-existing VEGF levels are unaffected, indicating that the CMT inhibits release of new VEGF rather than facilitating destruction of VEGF.
- Demonstrated that augmentation of released levels of VEGF by CMT-300 and diminution of released levels of VEGF by CMT-308 in MCF-7 cells as well as MDA-MB-435S cells is not accompanied by any significant alteration in levels of intracellular VEGF isoforms or mRNA species coding for those isoforms in either cell line. This result suggests that the two CMTs affect a post-transcriptional and post-translational step, possibly at the level of secretion.
- Further characterized the dose dependence of the effects of CMT-300 on VEGF release from Mono Mac 6 cells: 5 μM CMT-300 stimulates VEGF release with ~40% cytotoxicity and while higher doses are somewhat more cytotoxic, VEGF release appears to be inhibited in a dose dependent fashion which may reflect effects on secretion.
- Confirmed the dose-dependence of inhibition of VEGF release from Mono Mac 6 cells by CMT 308: 5 μM CMT-308 suppresses VEGF release by >50%, while 20 μM CMT-308 suppresses VEGF release by >90%, all in the absence of marked cytotoxicity. The release of VEGF and its suppression by CMT-308 are unresponsive to TGF-β.
- Characterized the time course of VEGF release from Mono Mac 6 cells and its suppression by CMT-308: demonstrated that newly released VEGF is first detected ~4 hours after initiation of culture of Mono Mac 6 cells, and that 20 μM CMT-308 suppresses release of VEGF by >90% for up to 48 hours.
- Demonstrated the effects of CMTs on levels of intracellular VEGF isoforms and mRNAs for those isoforms in Mono Mac 6 cells: CMT-300 diminishes intracellular levels of the 46 kDa isoform of VEGF as well as mRNAs coding for 121 amino acid and 165 amino acid species of VEGF, while CMT-308 has only minimal effects on the levels of the intracellular levels of VEGF isoforms or the mRNA species for those isoforms.
- Demonstrated that CMT-300 and CMT-308 diminish tube-like bridge formation in human microvascular endothelial cells cultured on plastic with an overlay of type I collagen. The effects are less pronounced than those which were previously observed in endothelial cells cultured on thick layers of Matrigel, but are qualitatively similar, with significant diminution in the number of bridges and the thickness of the remaining bridges at 30 μM doses of either CMT.
- Demonstrated that CMT-300 and CMT-308 inhibit endothelial cell migration through fibronectin-coated porous membranes in response to the chemotactic stimuli of VEGF alone or VEGF+serum. Inhibition of migration by CMT-300 is more effective than by CMT-308, and is more effective when VEGF alone is employed as the chemoattractant.

D. Reportable Outcomes

During September 2002, we presented our poster on "Antiangiogenic Action of Chemically Modified Tetracyclines in Breast Cancer" at the Department of Defense Breast Cancer Research Program Meeting "Era of Hope" [10]. We are now actively preparing two manuscripts suitable for publication, the first describing the effects of CMTs on multiple breast tumor cell lines and Mono Mac 6 cells and the second summarizing the effects of these tetracycline derivatives on endothelial cells.

E. Conclusions

In this third year of research, we have increased our understanding of the pleiotropic effects of two chemically modified tetracyclines, CMT-300 and CMT-308, on *in vitro* human cell-based models of aspects of tumor angiogenesis. The effects on inhibition of morphogenic changes by endothelial cells and on inhibition of endothelial cell migration in the absence of cytotoxicity to the cells suggest that a major mechanism by which the chemically modified tetracycline CMT-300 induces regression of the angioproliferative lesions in patients with Kaposi's Sarcoma may involve inhibition of the endothelial response to angiogenic signals. A similar effect of CMT-308 on endothelial cells may be combined with inhibition by this CMT of release of VEGF release from tumor cells and infiltrating macrophages, apparently at a post-transcriptional and post-translational step, possibly at the level of the secretory process. These results offer promise that the nonphototoxic chemically modified tetracycline CMT-308 may be even more effective than CMT-300 as an antiangiogenic agent in management of breast cancer.

F. References

1. Cianfrocca, M, Cooley, TP, Lee, JY, Rudek, MA, Scadden, DT, Ratner, L, Pluda, JM, Figg, WD, Krown, SE, and Dezube, BJ (2002) Matrix metalloproteinase inhibitor COL-3 in the treatment of AIDS-related Kaposi's sarcoma: a phase I AIDS melignancy consortium study. J. Clin. Oncol. 20:153-159.

2. Seftor, RE, Seftor, EA, Kirschmann, DA, and Hendrix, MJ (2002) Targeting the tumor microenvironment with chemically modified tetracyclines: inhibition of laminin 5 gamma2 chain promigratory fragments and vasculogenic mimicry. Mol. Cancer Therapeut. 1:1173-1179.

3. Fife, RS, Sledge, GW Jr, Sissons, S, and Zerler, B (2000) Effects of tetracyclines on angiogenesis *in vitro*. Cancer Lett. 153:75-78.

4. Rudek, MA, Figg, WD, Dyer, WV, Dahut, W, Turner, ML, Steinberg, SM, Liewehr, DJ, Kohler, DR, Pluda, JM, and Reed, E (2001) Phase I clinical trial of oral COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic cancer. J. Clin. Oncol. 19:584-592.

5. Zajchowski, DA, Bartholdi, MF, Gong, Y, Webster, L, Liu, H-L, Munishkin, A, Beauheim, C, Harvey, S, Ethier, SP, and Johnson, PH (2001) Identificaiton of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res. 61:5168-5178.

6. Price, JE, and Zhang, RD (1990) Studies of human breast cancer metastasis using nude mice. Cancer Metastasis Rev. 8:285-297.

7. Munaut, C, Noel, A, Hougrand, O, Foidart, J-M, Boniver, J, and Deprez, M (2003) Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2, and MMP-9 in human glioblastomas. Int. J. Cancer 106:848-855.

8. Kubota, Y, Kleinman, HK, Martin, GR, and Lawley, TJ (1988) Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J. Cell Biol. 107:1589-1598.

9. Whelan, MC, and Senger, DR (2003) Collagen I initiates endothelial cell morphogenesis by inducing actin polymerization through suppression of cyclic AMP and protein kinase A. J. Biol. Chem. 278:327-334.

10. Simon, SR, and Kothari, M (2002) Antiangiogenic action of chemically modified tetracyclines in breast cancer. Department of Defense Breast Cancer Research Program Meeting: Era of Hope. Proceedings, Vol III, P46-19.

APPENDIX

Figures

Figure 1. Effects of CMTs on MDA-MB-435S cells cultured in the absence of TGF-β. Cells were plated in 48 well plates at a density of 10^5 cells per well. After culture overnight in complete medium, the cells were incubated for an additional 24 hours at 37°C in fresh serum-free medium containing 0, 1, 5, 10, 20, 30, or 50 μM CMT-300 or CMT-308. After the 24 hour incubation in serum-free medium, the supernatants were collected for assay of VEGF by ELISA and replaced with a solution of MTS, followed by an additional incubation for 1 hour. Mitochondrial dehydrogenase activity, a measure of cell viability, was detected by recording absorbance of the formazan product of MTS in a microplate reader at 490 nm.. The results are the means of four independent experiments.

Figure 2. Effects of CMTs on MCF-7 cells stimulated with 1 ng/ml TGF-β. Cells were plated in 48 well plates and, after overnight culture in complete medium, were transferred to serum-free medium containing TGF-β and/or CMT-300 or CMT-308 as in Figure 1. Supernatants were collected for assay of VEGF by ELISA and mitochondrial dehydrogenase activity of the cells was then measured with MTS as in Figure 1.

Figure 3. Time course of release of VEGF from MCF-7 cells in the absence and presence of CMT-308. Cells were plated in 48 well plates and, after overnight culture in complete medium, were transferred to serum-free medium in the presence or absence of 20 μM CMT-308. The plates were incubated at 37°C for 8 hours, at which time supernatant medium was collected from some wells of cells cultured under both conditions. 20 μM CMT-308 was then added to other wells of cells which had been cultured in the absence of the tetracycline derivative and at the end of an additional 8 hours, supernatants were removed from all the wells, including those cells which had received CMT 16 hours earlier at the start of the experiment and those cells which had received no CMT over the entire time course. Supernatants collected at the end of the first 8 hours and the second 8 hours were all assayed for VEGF by ELISA.

Figure 4. Effects of CMT-300, CMT-308, and TGF-β on VEGF release from MCF-7 cells. Cells were plated in 48 well plates and, after overnight culture in complete medium, were transferred to serum-free medium containing the indicated concentrations of TGF-β alone or in the presence of either 5 μM CMT-300 or 20 μM CMT-308 for 24 hours at 37°C. Supernatants were collected for assay of VEGF by ELISA as in Figure 1.

Figure 5. Effects of CMT-300, CMT-308, and TGF-β on VEGF release from MDA-MB-435S. Cells were plated in 48 well plates and, after overnight culture in complete medium, were transferred to serum-free medium containing the indicated concentrations of TGF-β alone or in the presence of either 5 μM CMT-300 or 20 μM CMT-308 for 24 hours at 37°C. Supernatants were collected for assay of VEGF by ELISA as in Figure 1.

Figure 6. Effects of CMT-300, CMT-308, and TGF-β on pool size of intracellular VEGF species in MCF-7 cells. Cells were plated in 48 well plates and, after overnight culture in complete medium, were transferred to serum-free medium alone or containing the indicated concentrations

of either 5 μM CMT-300 or 20 μM CMT-308 in the absence or presence of 1 ng/ml TGF-β for 24 hours at 37°C. The supernatants were removed and the cells lysed in a buffer containing protease inhibitors (Roche Biochemicals) by three cycles of freezing and thawing. After removal of insoluble cell debris, the lysates were measured for total protein content by BCA (Pierce). Equivalent protein concentrations were loaded onto 10% polyacrylamide gels and separated by electrophoresis in Bis-Tris buffer containing SDS. The proteins in the gels were transferred to PVDF membranes, probed with anti-VEGF antibodies, and immunoreactive bands visualized with an ECL Advanced kit (Amersham).

Figure 7. Effects of CMT-300, CMT-308, and TGF-β on pool size of intracellular VEGF species in MDA-MB-435S cells. Cells were plated in 48 well plates and, after overnight culture in complete medium, were transferred to serum-free medium alone or containing the indicated concentrations of either 5 μM CMT-300 or 20 μM CMT-308 in the absence or presence of 1 ng/ml TGF-β for 24 hours at 37°C. The supernatants were removed, the cells lysed, and the lysates analyzed for VEGF and actin as described in Figure 6.

Figure 8. Effects of CMT-308 and TGF-β on pool sizes of mRNA species for VEGF in MCF-7 cells. Cells were cultured for 24 hours in the absence or presence of 1 ng/ml TGF-β containing different concentrations of CMT-308 as described in Figure 2 and after removal of the supernatants, total RNA was isolated using TRI reagent (Molecular Research Center). mRNA species coding for VEGF isoforms of 189 amino acids, 165 amino acids, 145 amino acids, and 121 amino acids were amplified by PCR using the following primer pairs: 5'-CTGGTGGACATCTTCCAGGAGTA-3' and 5'-CTCACCGCCTCGGCTTGTCACA-3'. A human glyceraldehyde-3-phosphate dehydrogenase primer set (Clontech) was used to normalize concentrations of amplified products applied to agarose gels.

Figure 9. Effects of CMT-300, CMT-308 and TGF-β on pool sizes of mRNA species for VEGF in MCF-7 cells. Cells were cultured for 24 hours in the absence or presence of 1 ng/ml TGF-β containing 5 μM CMT-300 or 20 μM CMT-308 as described in Figure 2 and after removal of the supernatants, total RNA was isolated and the mRNA species coding for multiple VEGF isoforms were amplified with the primers described in Figure 8 along with primers for glyceraldehyde-3-phosphate dehydrogenase.

Figure 10. Effects of CMT-300, CMT-308 and TGF-β on pool sizes of mRNA species for VEGF in MCF-7 cells. Cells were cultured for 24 hours in the absence or presence of 1 ng/ml TGF-β containing 5 μM CMT-300 or 20 μM CMT-308 as described in Figure 2 and after removal of the supernatants, total RNA was isolated and the mRNA species coding for multiple VEGF isoforms were amplified with the primers described in Figure 8 along with primers for glyceraldehyde-3-phosphate dehydrogenase.

Figure 11. Effects of CMTs on viability and release of VEGF from Mono Mac 6 cells. Cells were plated directly in serum-free medium at a density of 10^5 per well in 48 well plates and sets of quadruplicate wells were then immediately treated with 0, 5, 10, or 20 μM CMT-300 or CMT-308. After 24 hours of incubation at 37°C, supernatant medium was collected from duplicate wells for assay of VEGF by ELISA and the remaining duplicate wells were incubated with MTS

reagent for an additional hour for assay of viability. Conversion of MTS to its formazan was recorded on a microplate reader at 490 nm.

Figure 12. Time course of release of VEGF from Mono Mac 6 cells in the absence and presence of CMT-308. Cells were plated directly in serum-free medium at a density of 10^5 per well in 48 well plates in the absence or presence of 20 μM CMT-308. The plates were incubated at 37°C and at the indicated times, supernatants were removed from duplicate wells for assay of VEGF by ELISA as in Figure 11.

Figure 13. Insensitivity of Mono Mac 6 cells to TGF-β. Cells were plated directly in serum-free medium at a density of 10^5 per well in 48 well plates and sets of quadruplicate wells were then immediately treated with the indicated concentrations of TGF-β in the absence or presence of 5 μM CMT-300 or 20 μM CMT-308. After 24 hours of incubation at 37°C, supernatant medium was collected from duplicate wells for assay of VEGF by ELISA.

Figure 14. Effects of CMT-300 and CMT-308 on pool size of intracellular VEGF species in Mono Mac 6 cells. Cells were plated directly in serum-free medium at a density of 10^5 per well in 48 well plates in the absence or presence of 5 μM CMT-300 or 20 μM CMT-308. The plates were incubated at 37°C for 24 hours. The supernatants were removed, the cells lysed, and the lysates as well as the supernatant medium analyzed for VEGF and actin as described in Figure 6.

Figure 15. Effects of CMT-300 and CMT-308 on pool sizes of mRNA species for VEGF in Mono Mac 6 cells. Cells were cultured for 24 hours in the absence or presence of 5 μM CMT-300 or 20 μM CMT-308 as described in Figure 14 and after removal of the supernatants, total RNA was isolated and the mRNA species coding for multiple VEGF isoforms were amplified with the primers described in Figure 8 along with primers for glyceraldehyde-3-phosphate dehydrogenase.

Figure 16. Effects of CMT-300 and CMT-308 on morphogenesis by human microvascular endothelial cells (HMVEC) after application of an overlay of type I collagen. Cells were maintained in 48 well plates for 24 hours in CMT-free medium or in medium containing 30 μM CMT-300 or CMT-308. The medium was then replaced with medium containing 500 μg/ml type I collagen purified from rat tail and the cells were cultured for an additional seven hours. The cells were then examined under a Nikon Diaphot microscope by phase contrast.

Figure 17. Failure of subsequent addition of CMT 300 to reverse morphogenic changes in human microvascular endothelial cells after application of an overlay of type I collagen. Cells were maintained in 48 well plates for 24 hours in CMT-free medium. The medium was then replaced with medium containing 500 μg/ml type I collagen purified from rat tail and the cells were cultured overnight. The medium was then replaced with medium containing 30 μM CMT-300 and the cells were cultured for an additional 24 hours. The wells were then gently washed with HBSS and stained with Calcein AM for 45 minutes at 37°C. The excess dye was washed off gently with HBSS and the wells were examined under a Nikon Diaphot microscope with epifluorescence (488 nm excitation; 515 nm emission).

Figure 18. Effects of subsequent addition of CMT 308 on reversal of morphogenesis by human microvascular endothelial cells after application of an overlay of type I collagen. Cells were maintained in 48 well plates for 24 hours in CMT-free medium. The medium was then replaced with medium containing 500 μg/ml type I collagen purified from rat tail and the cells were cultured overnight. The medium was then replaced with medium containing 30 μM CMT-308 and the cells were cultured for an additional 24 hours. The cells were stained with Calcein AM and visualized as in Figure 17.

Figure 19. Effects of CMT-300 on migration of endothelial cells through porous fibronectin coated membranes. Human umbilical vein endothelial cells (Cascade Biologics) were applied to fibronectin-coated porous Fluoro-Blok membrane inserts for 24-well plates (Becton Dickinson Biosciences) at a density of 5 x 10^4 cells/chamber in medium alone or containing 10 μM CMT-300. Serum free medium alone or containing 0.5, 2, or 10 ng/ml VEGF, or 10 ng/ml VEGF in 10% fetal bovine serum-containing medium was added to the wells below the inserts and the plates with their inserts were incubated at 37° for 22 hours. The medium was then removed from the inserts which were then transferred to a second 24-well plate containing 4 μg/ml Calcein AM (Molecular Probes). The plates with their inserts were incubated for an additional 90 minutes, and the fluorescence arising from those cells which had migrated through the inserts was measured with a Cytofluor 2300 microplate fluorimeter.

Figure 20. Effects of CMT-308 on migration of endothelial cells through porous fibronectin coated membranes. Human umbilical vein endothelial cells were applied to fibronectin-coated porous Fluoro-Blok membrane inserts for 24-well plates at a density of 5 x 10^4 cells/chamber in medium alone or containing 20 μM CMT-308. Serum free medium alone or containing 0.5, 2, or 10 ng/ml VEGF, or 10 ng/ml VEGF in 10% fetal bovine serum-containing medium was added to the wells below the inserts and the plates with their inserts were incubated at 37° for 22 hours. The medium was then removed from the inserts which were then transferred to a second 24-well plate containing 4 μg/ml Calcein AM. The plates with their inserts were incubated for an additional 90 minutes, and the fluorescence arising from those cells which had migrated through the inserts was measured with a Cytofluor 2300 microplate fluorimeter.

Effects of CMTs on Cytotoxicity and VEGF secretion by MDA-MB-435S cells

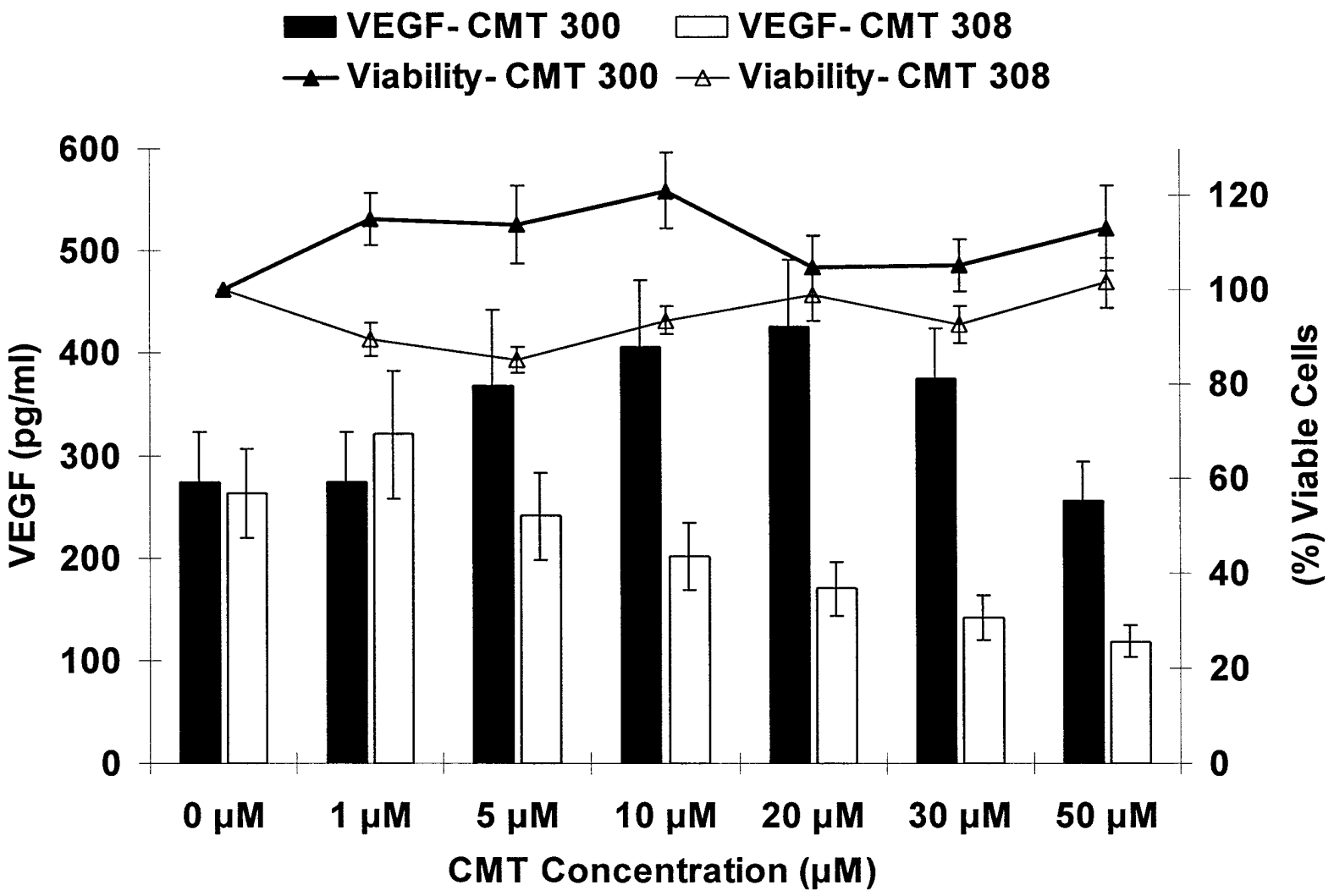


Fig 1

Effects of CMTs on Viability and VEGF secretion by MCF-7 cells

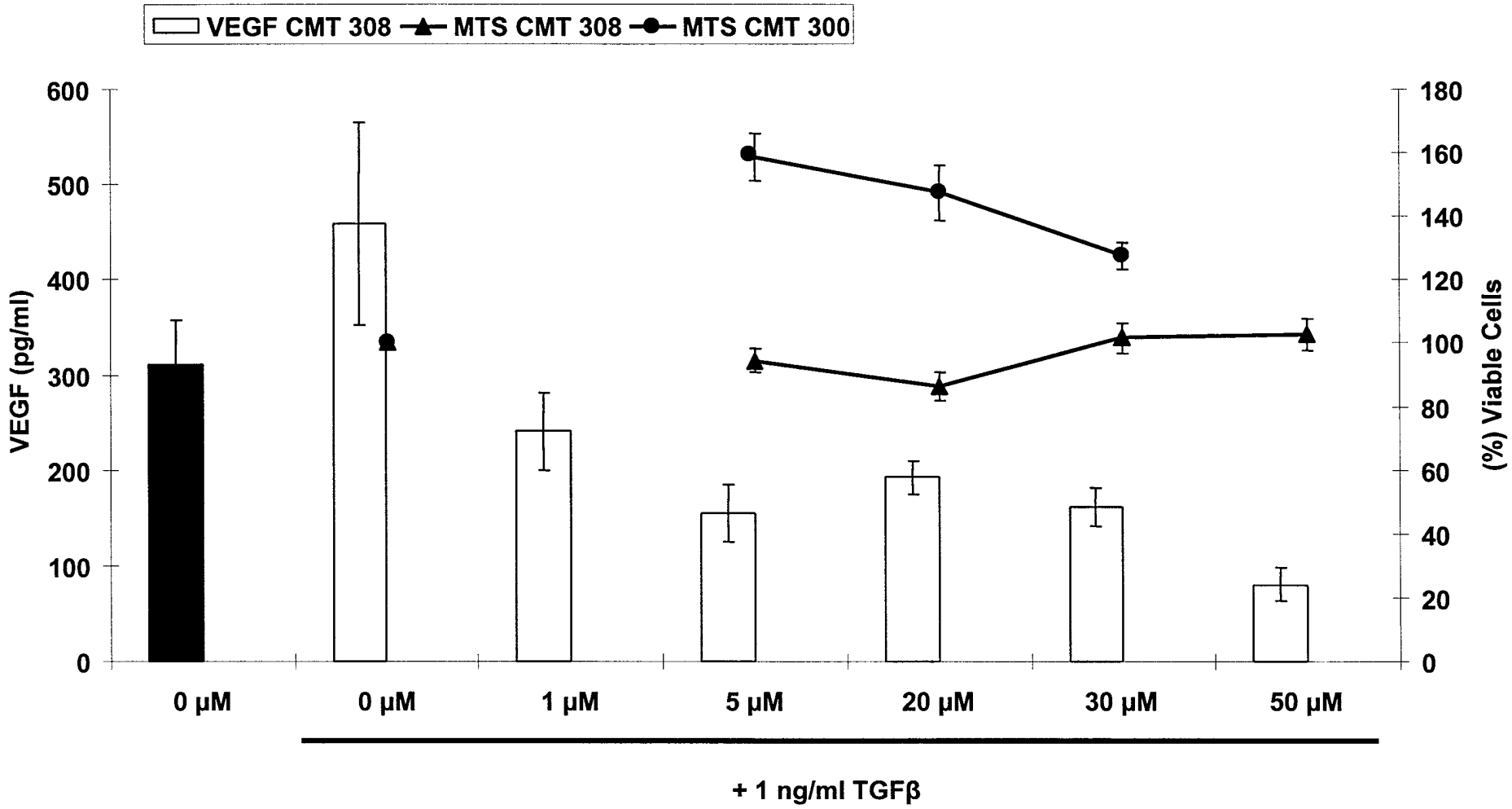


Fig 2

Duration of Suppression of VEGF Release by CMT-308 in MCF-7 cells

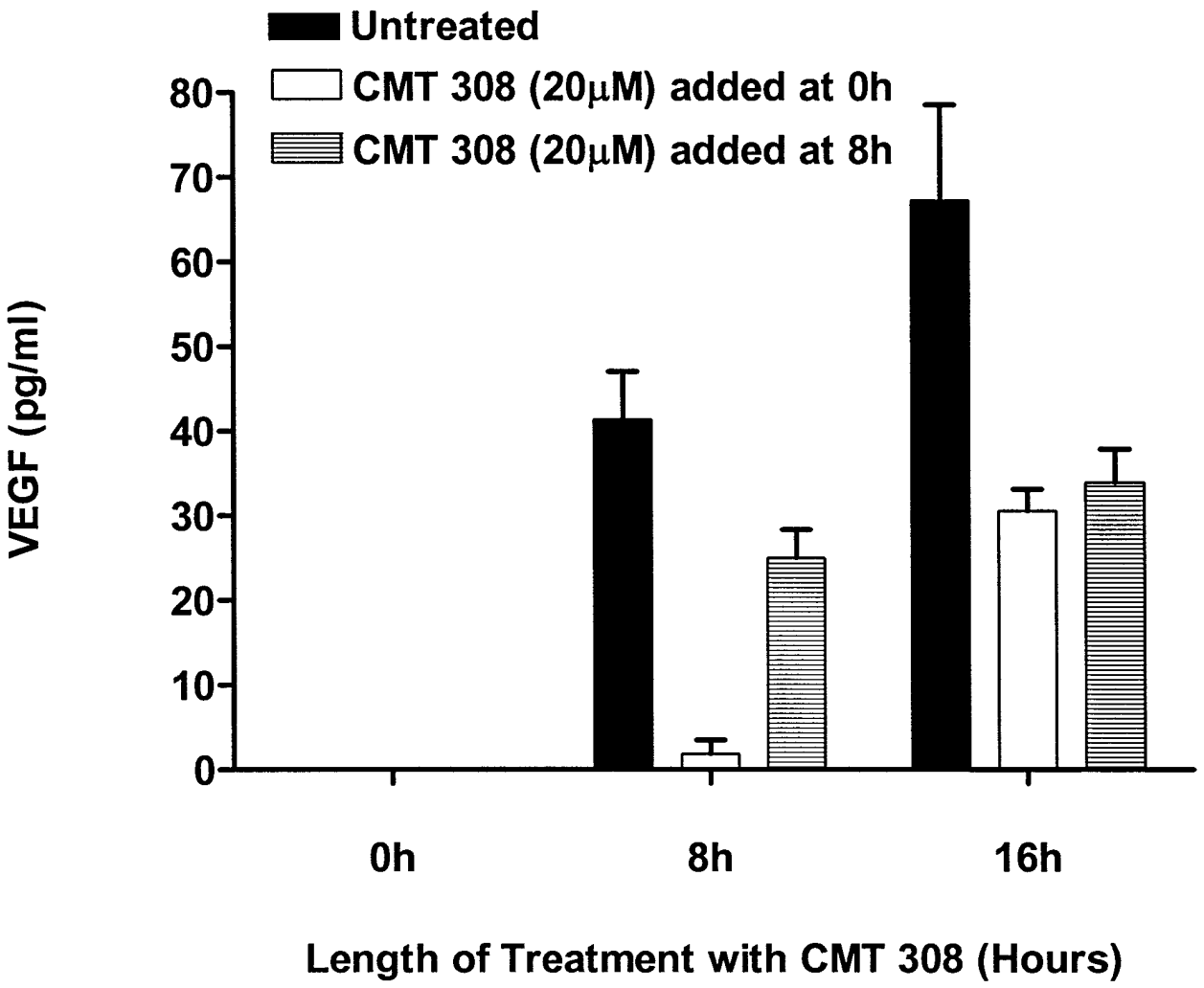


Fig 3

* = p<0.01 for Untreated vs CMT 308
# = p<0.001 for CMT 300 vs CMT 308

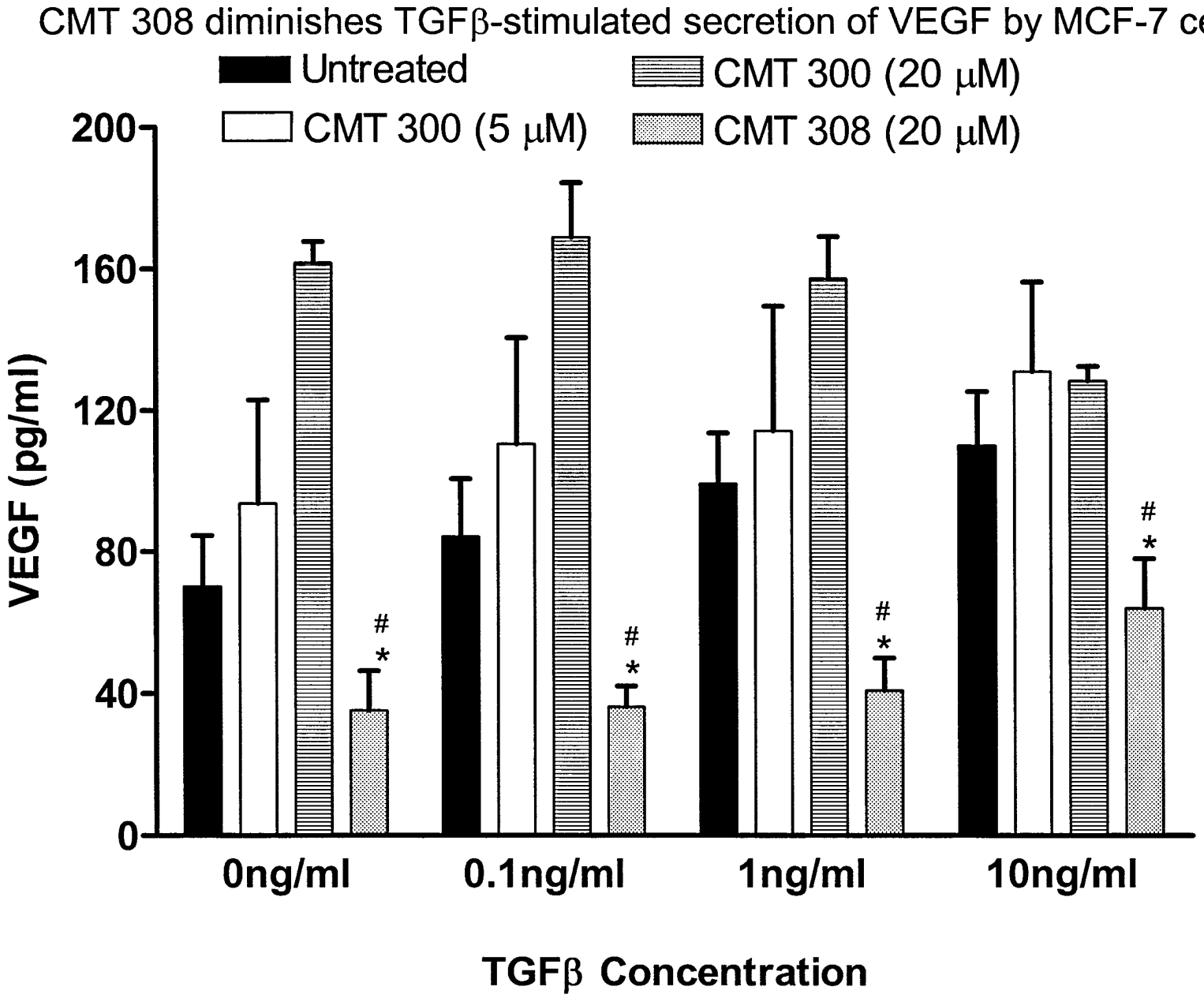


Fig 4

CMT 308 diminishes TGFβ-stimulated secretion of VEGF by MDA-MB-435S cells

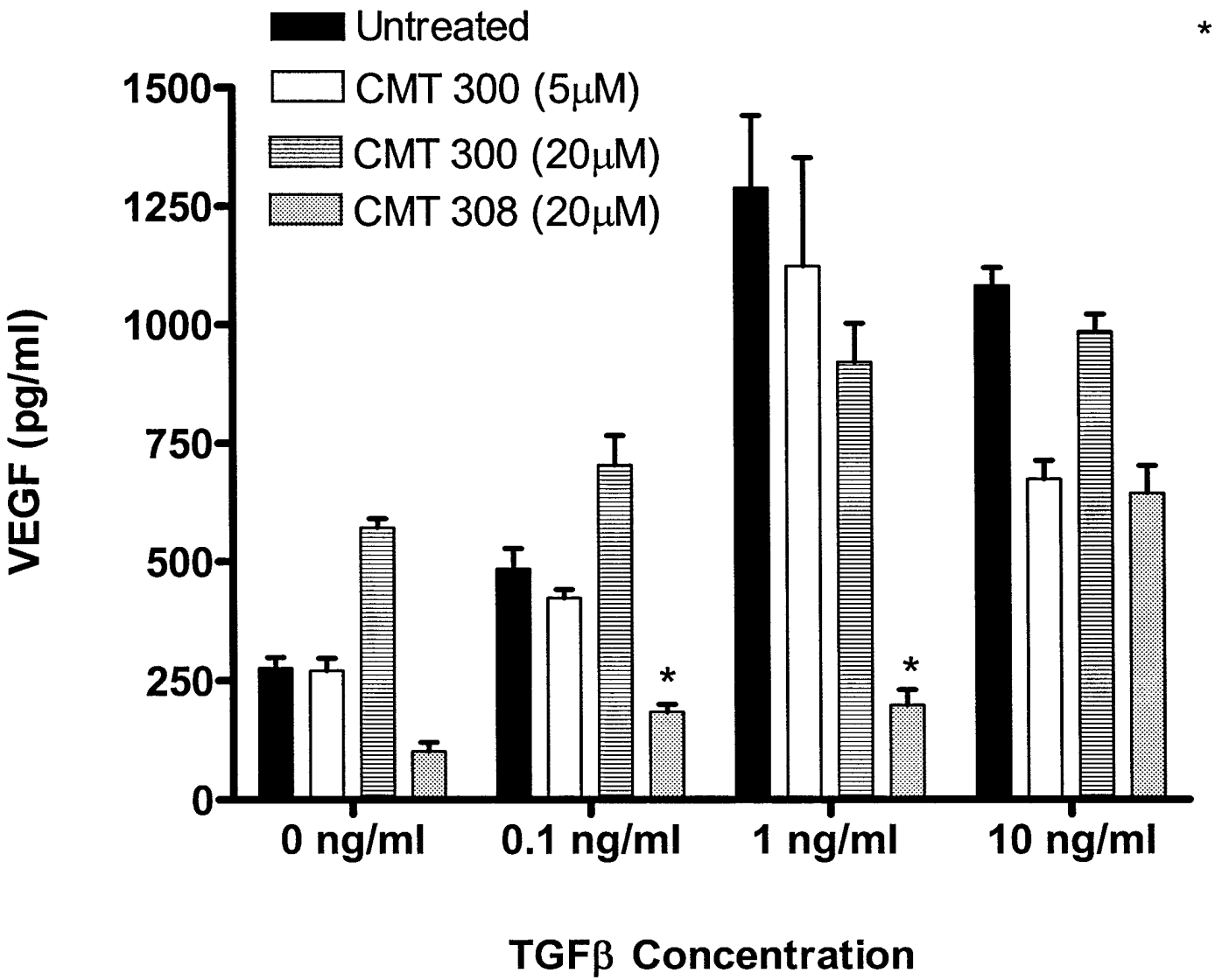


Fig 5

Effect of TGFβ and CMTs on intracellular levels of VEGF in MCF-7 cells

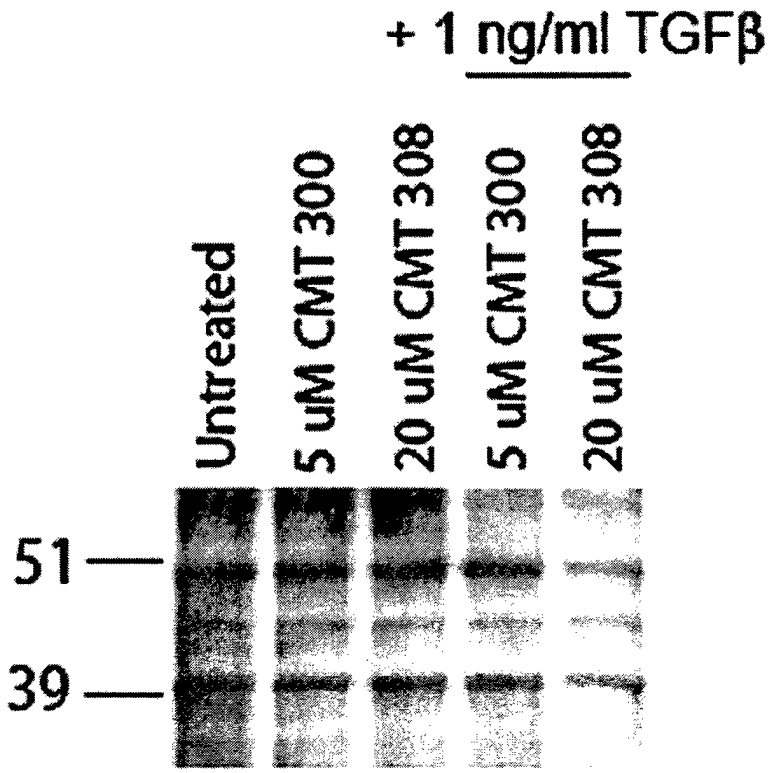


Fig 6

Effect of TGFβ and CMTs on intracellular levels of VEGF in MDA-MB-435S cells

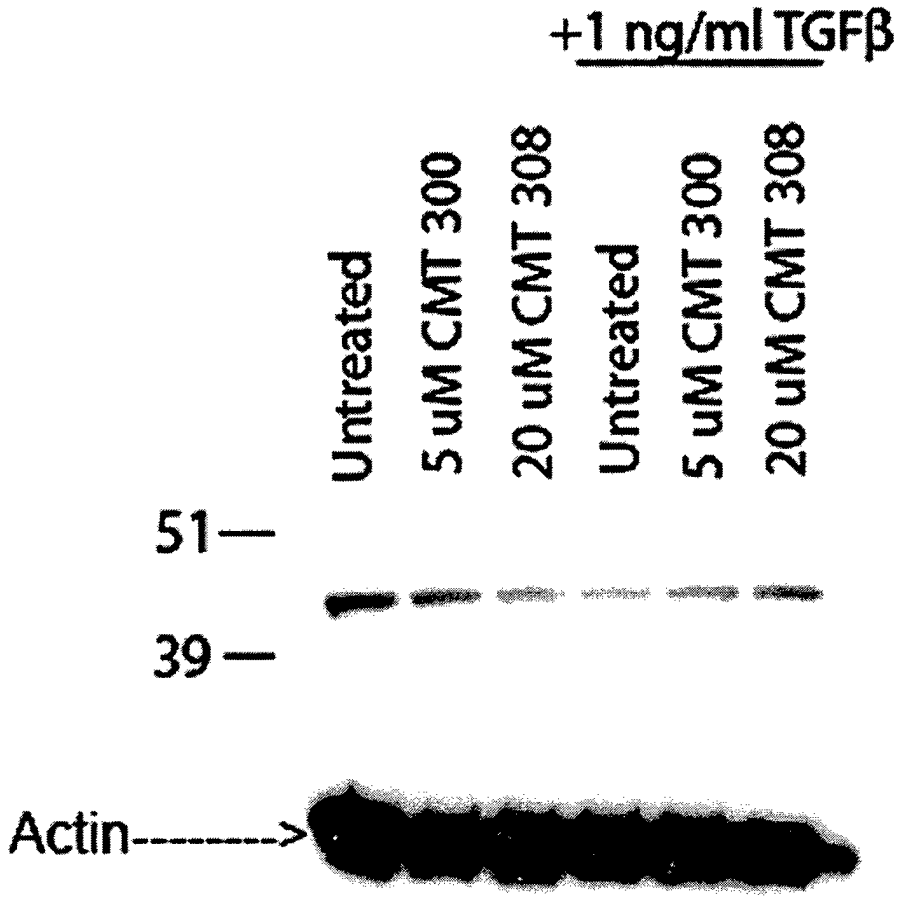


Fig 7

Effect of CMT 308 on VEGF mRNA in MCF-7 cells

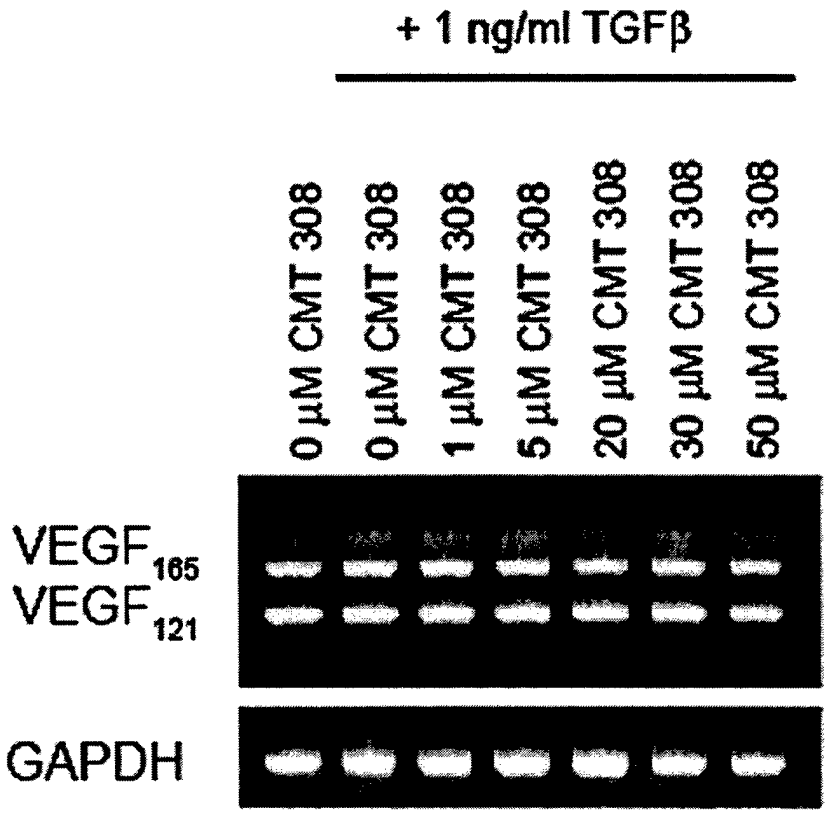


Fig 8

Effects of TGFβ and CMTs on VEGF mRNA in MCF-7 cells

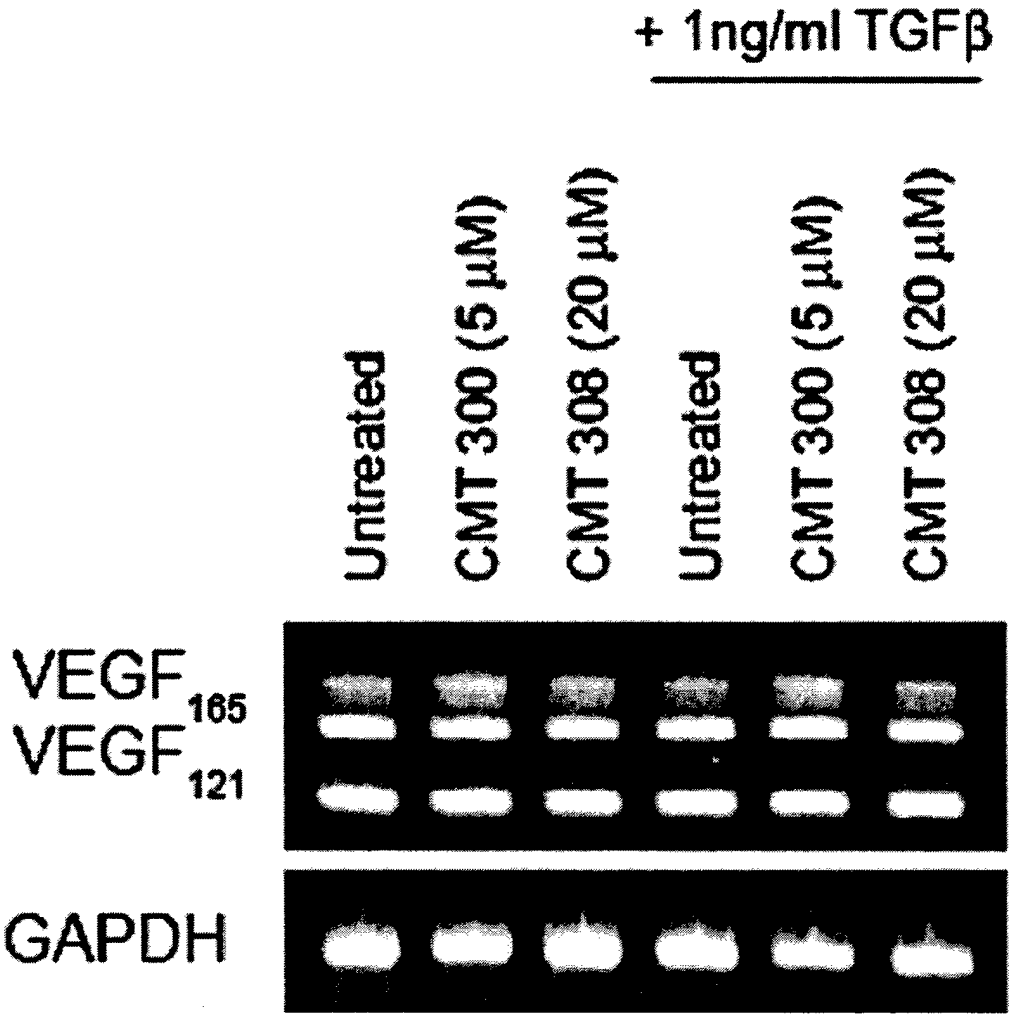


Fig 9

Effects of TGFβ and CMTs on VEGF mRNA in MDA MB 435S cells

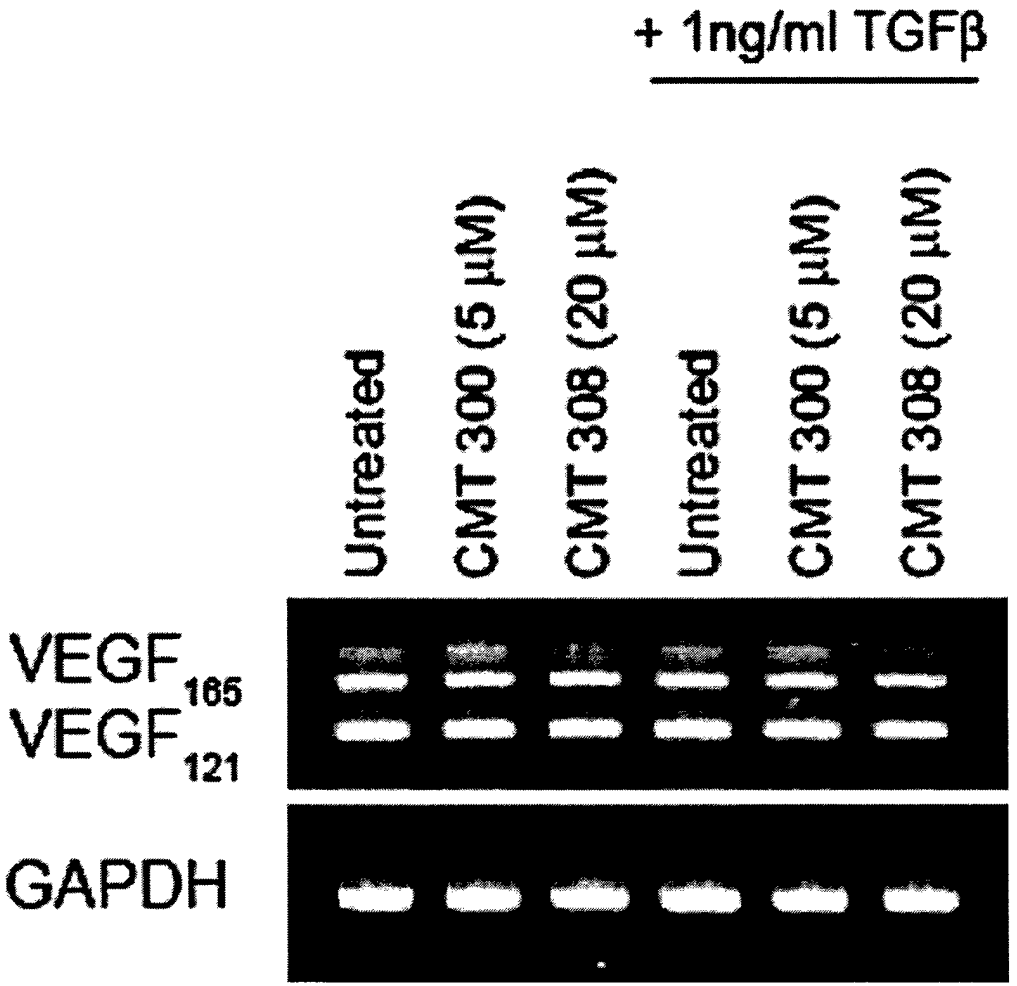


Fig 10

Cytotoxicity and Dose Response of Mono Mac 6 cells to CMTs

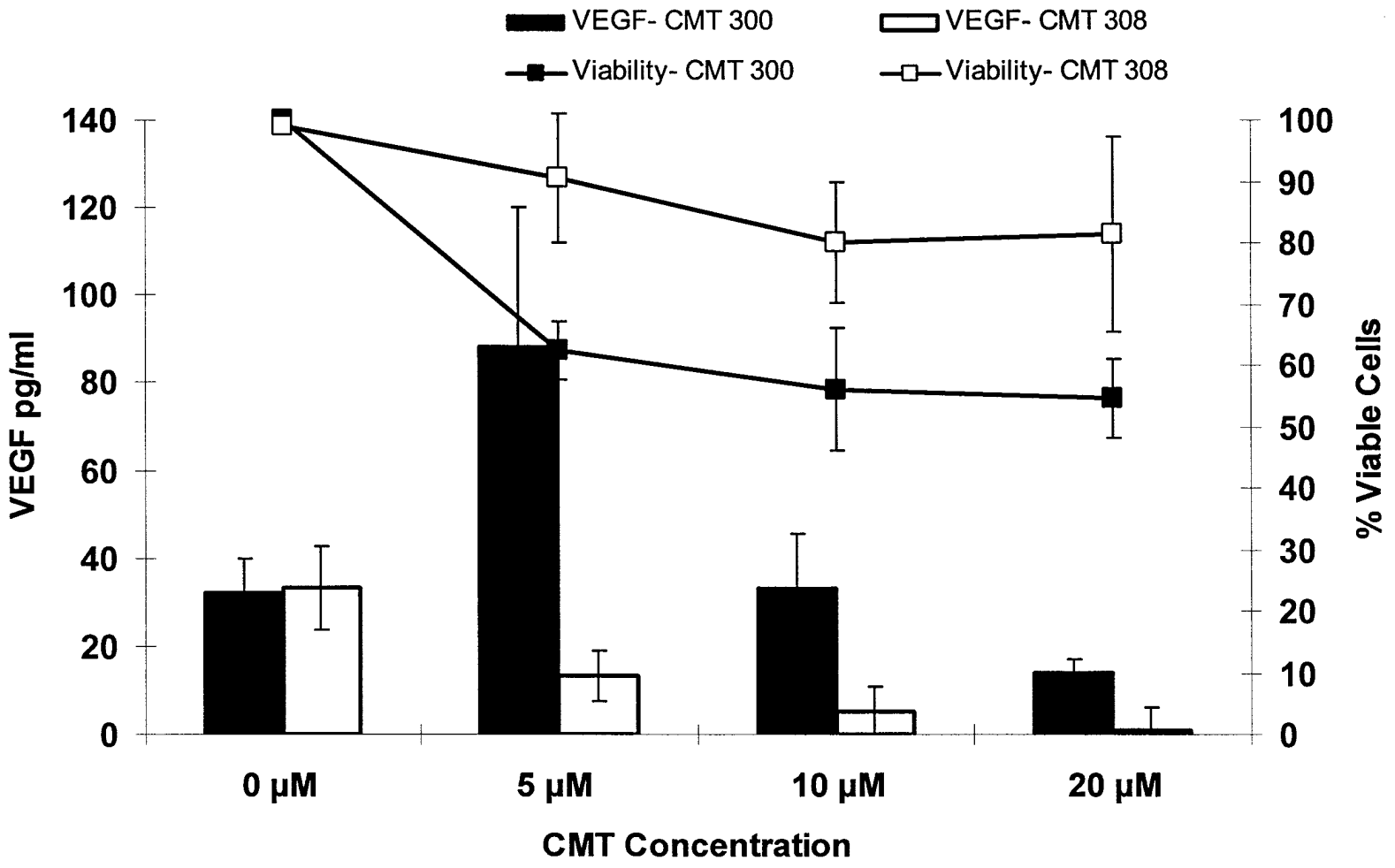


Fig 11

Duration of suppression of VEGF release by CMT-308 in Mono Mac 6 cells

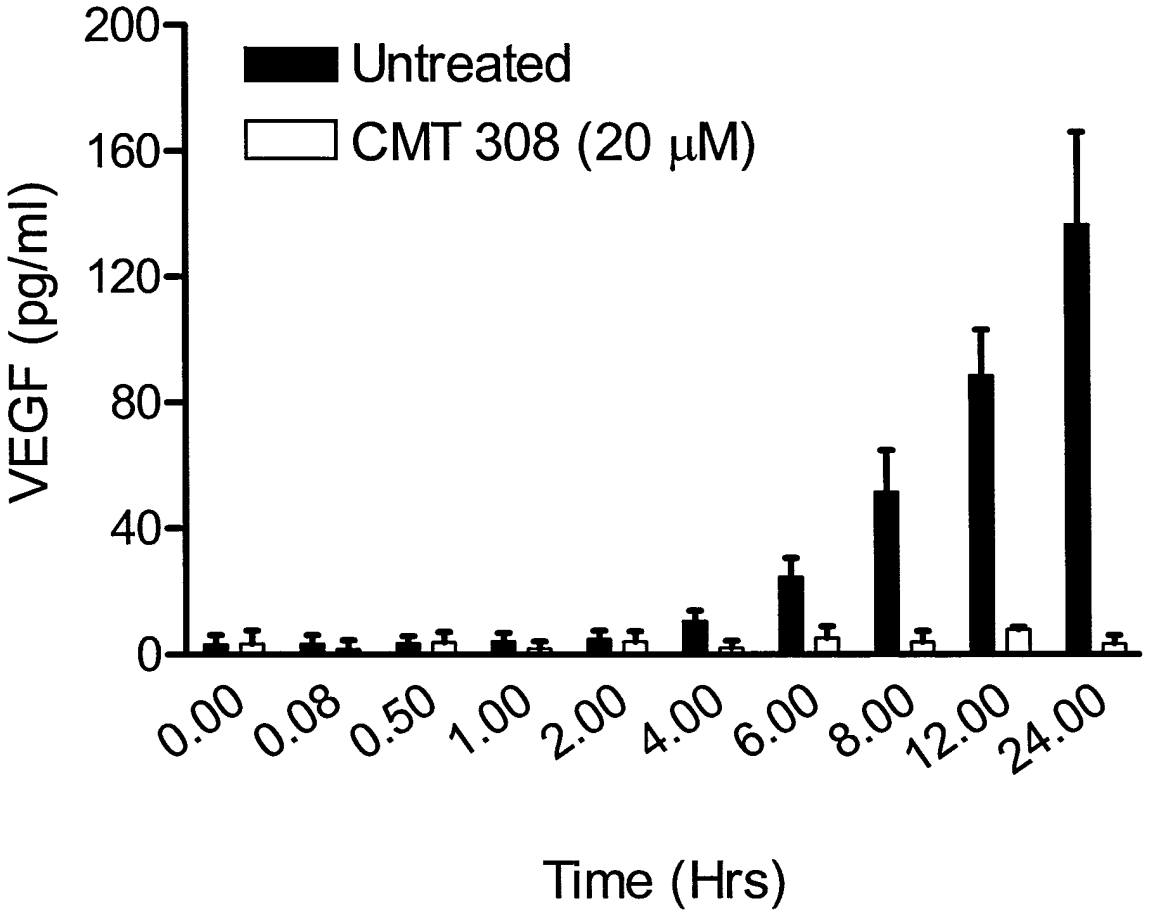


Fig 12

Effect of TGFβ and CMTs on VEGF secretion by Mono Mac 6 cells

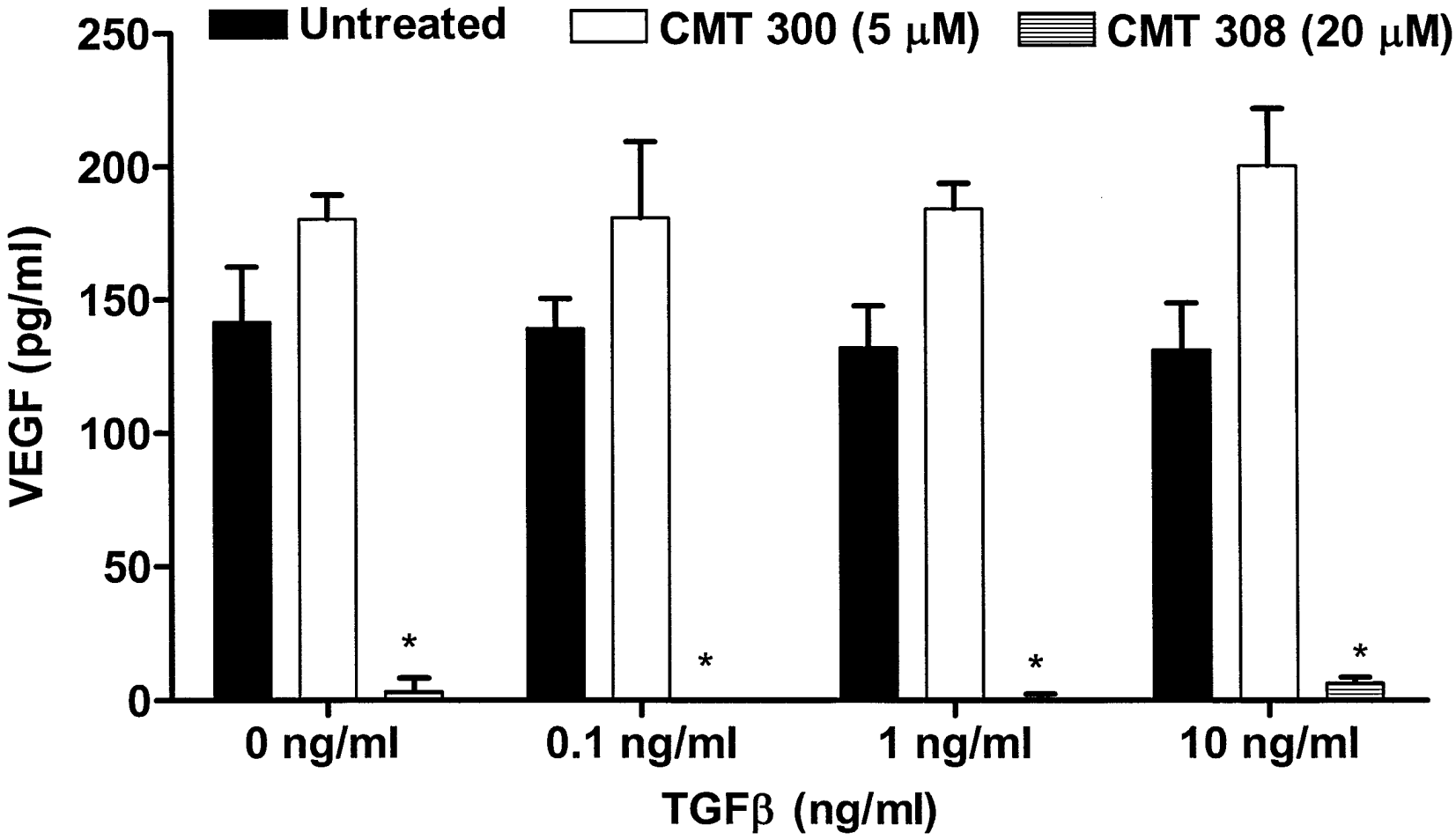


Fig 13

* p< 0.0001 1-way ANOVA

Effect of CMTs on levels of VEGF in Mono Mac 6 cells and their conditioned media

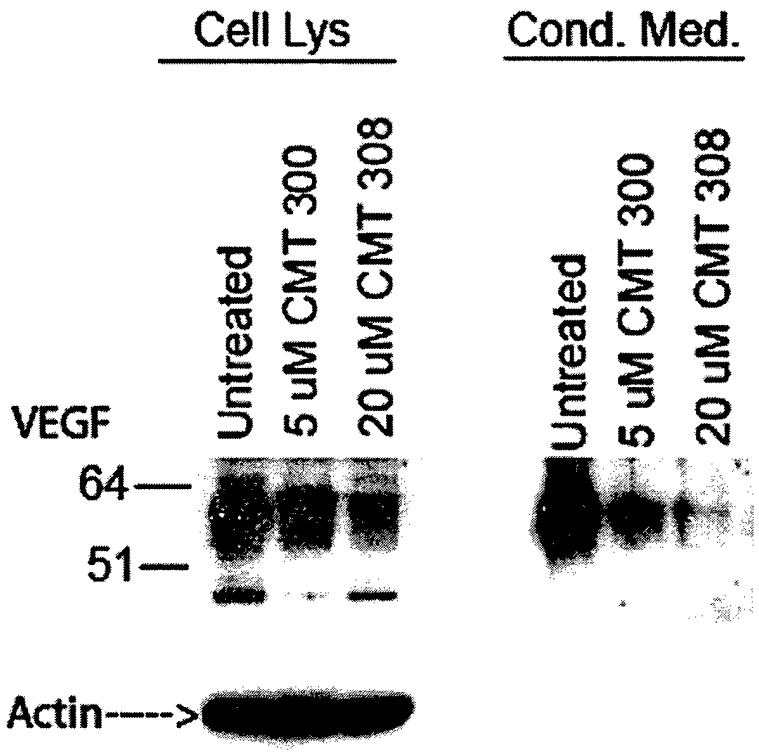


Fig 14

Effect of CMTs on VEGF mRNA in Mono Mac 6 cells

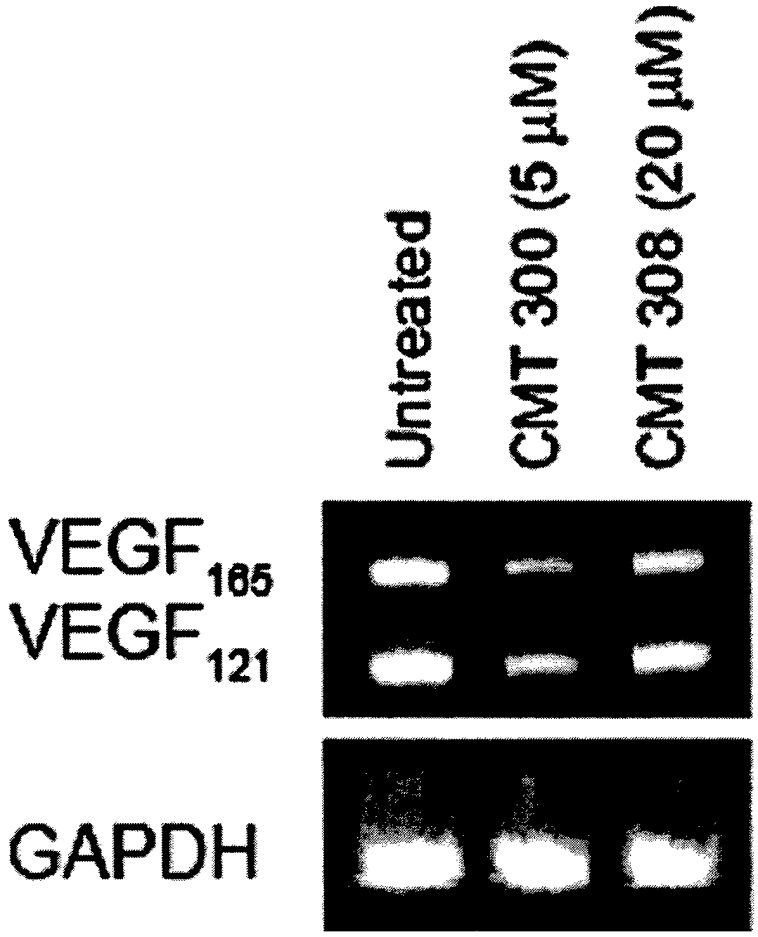
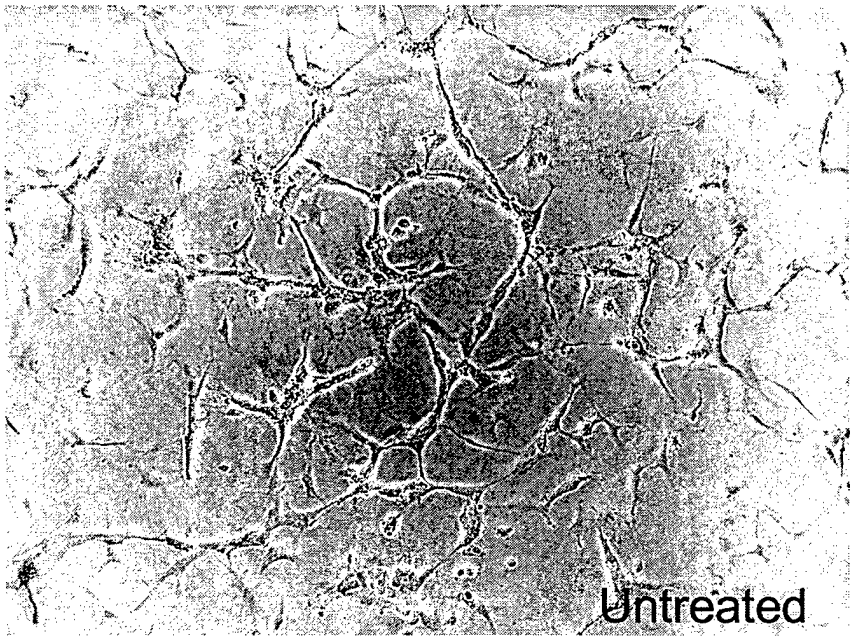
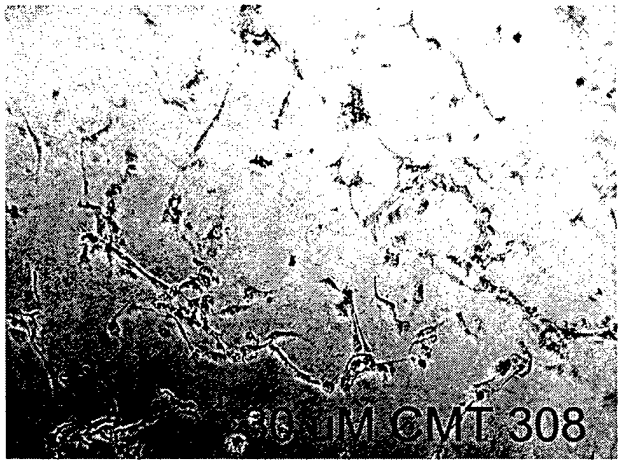


Fig 15

Effect of CMT 300 and 308 on morphogenesis induced by type I collagen on HMVEC





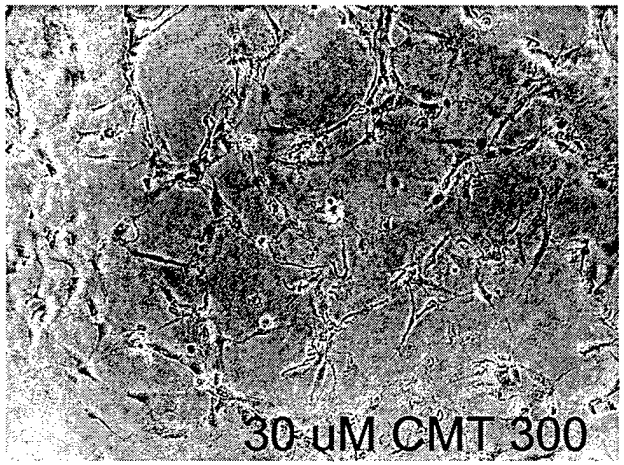
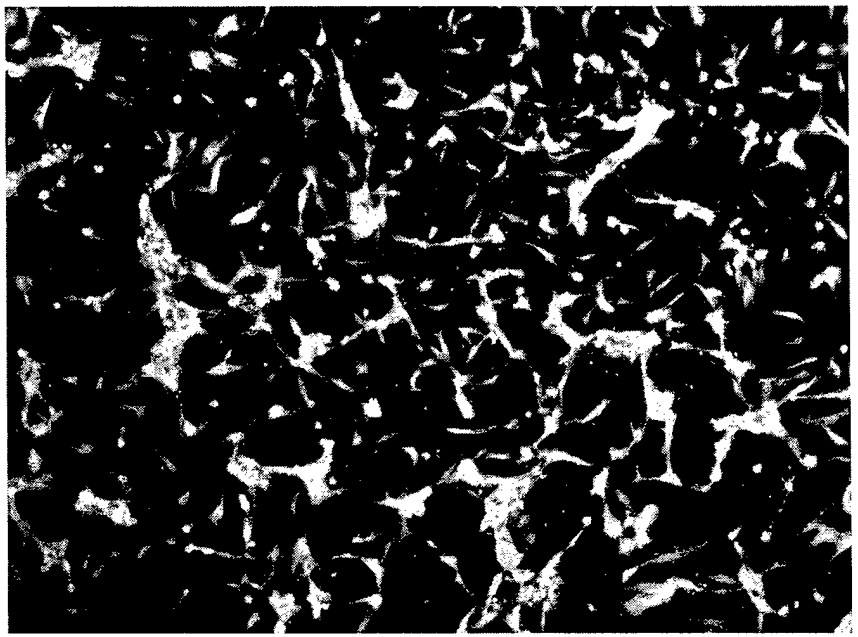
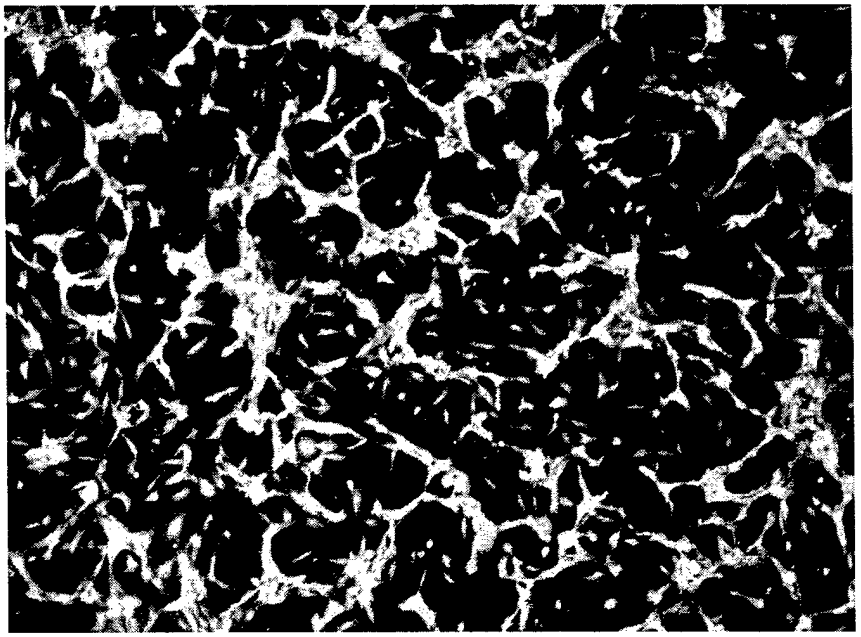


Fig 16

Effect of CMT 300 on Pre-formed HMVEC tubes



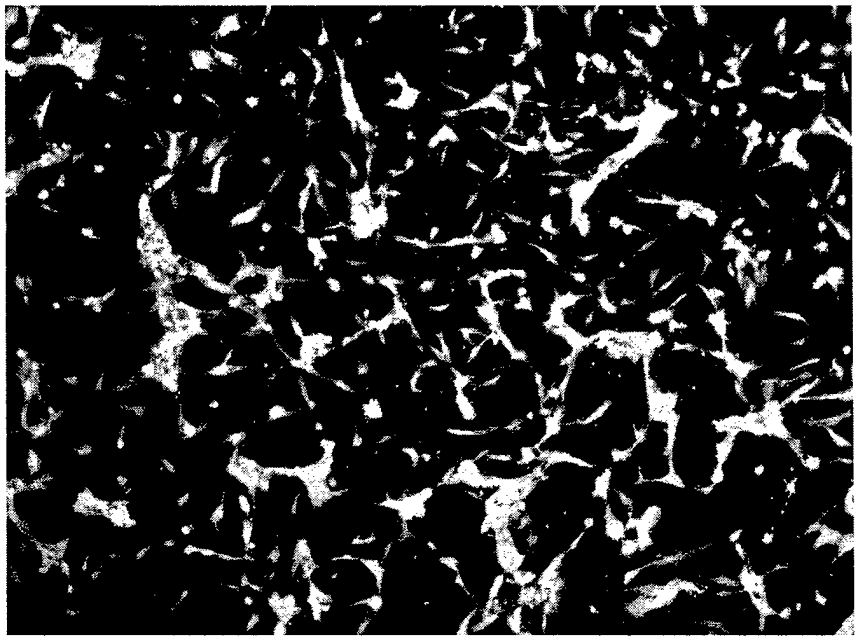
DMSO treated



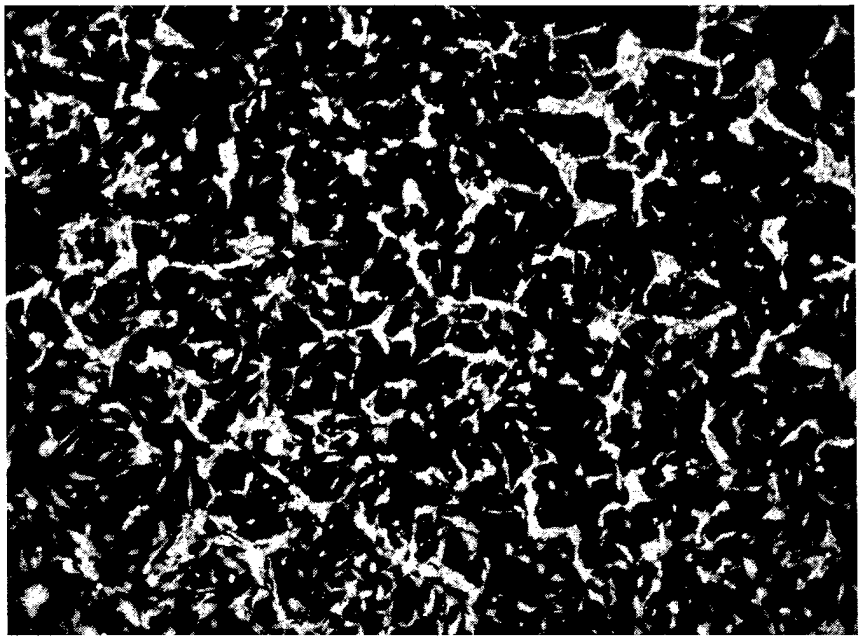
30 uM CMT 300 treated

Fig 17

Effect of CMT 308 on Pre-formed HMVEC tubes



DMSO treated



30 uM CMT 308 treated

Fig 18

Effect of CMT 300 on migration of HUVEC through fibronectin coated inserts

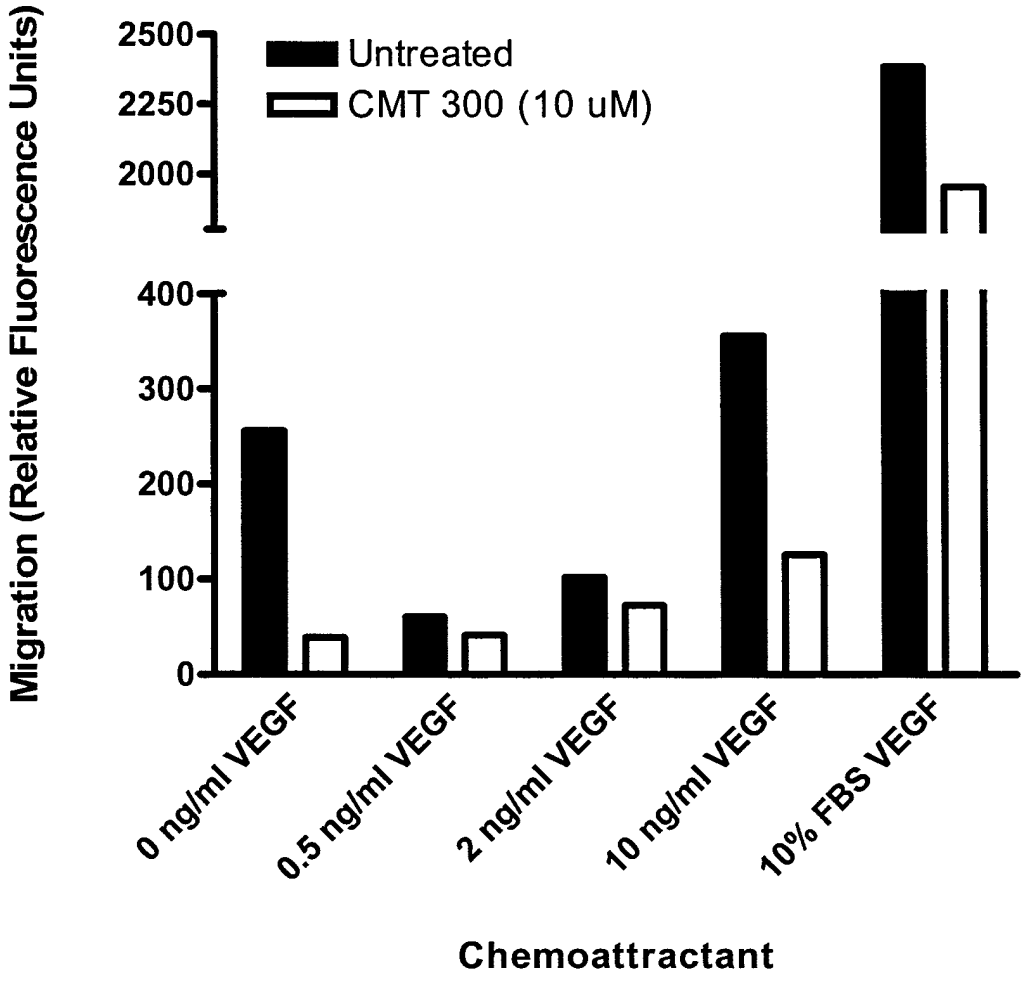


Fig 19

Effect of CMT 308 on migration of HUVEC through fibronectin coated inserts

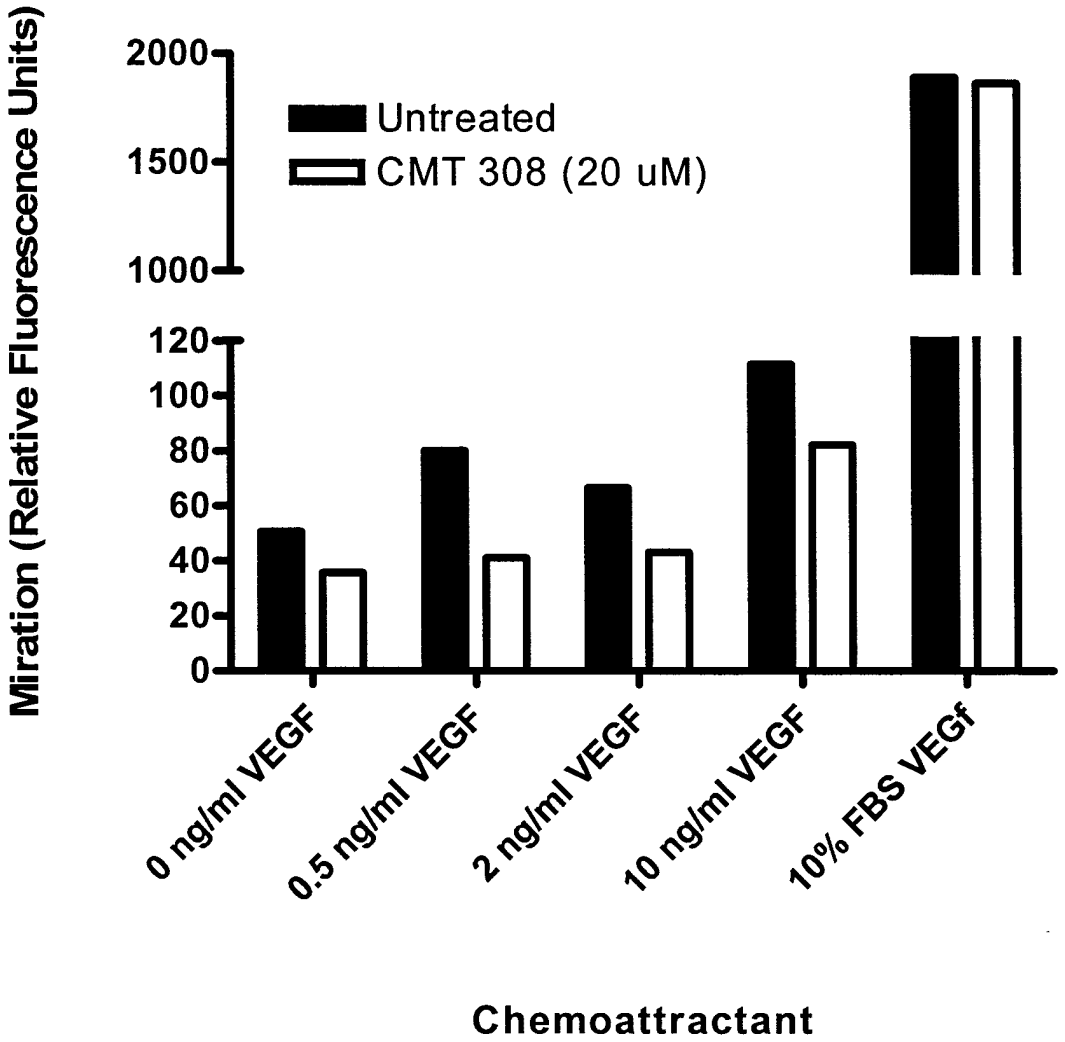


Fig 20